

68 fo 1

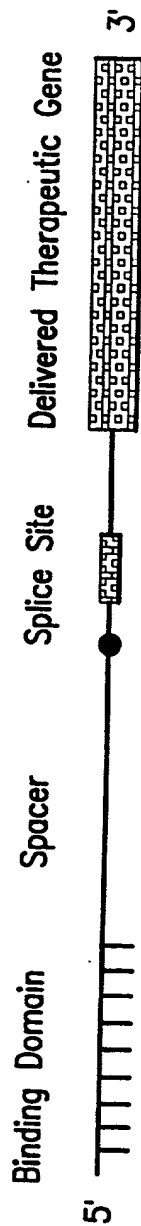


FIG. 1A

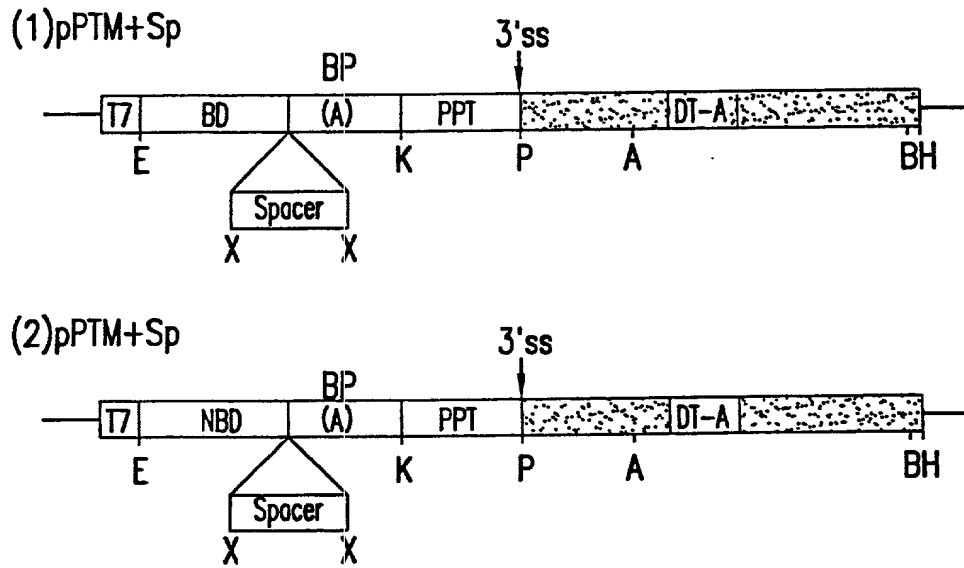


FIG.1B

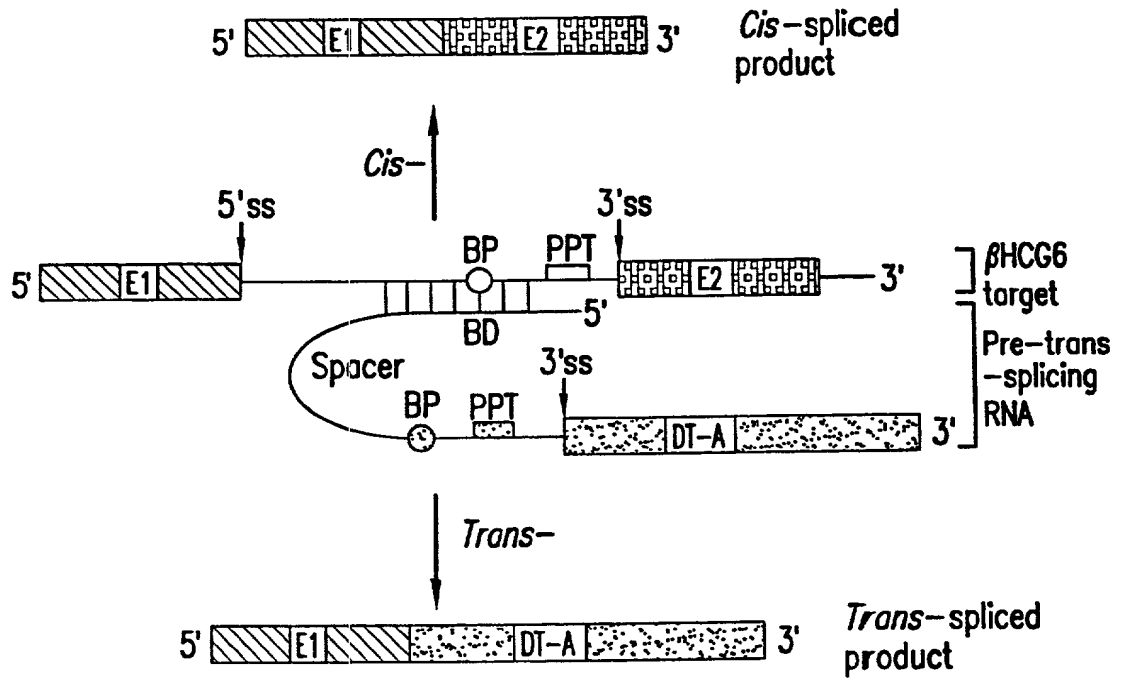


FIG.1C

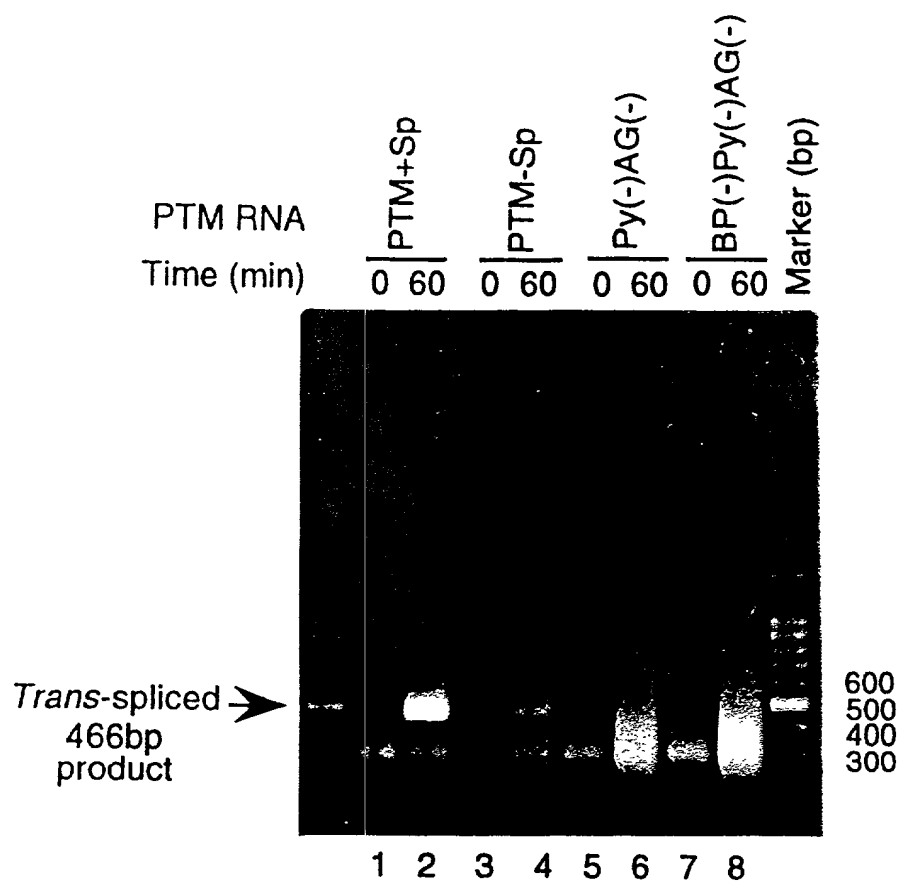


FIG.2A

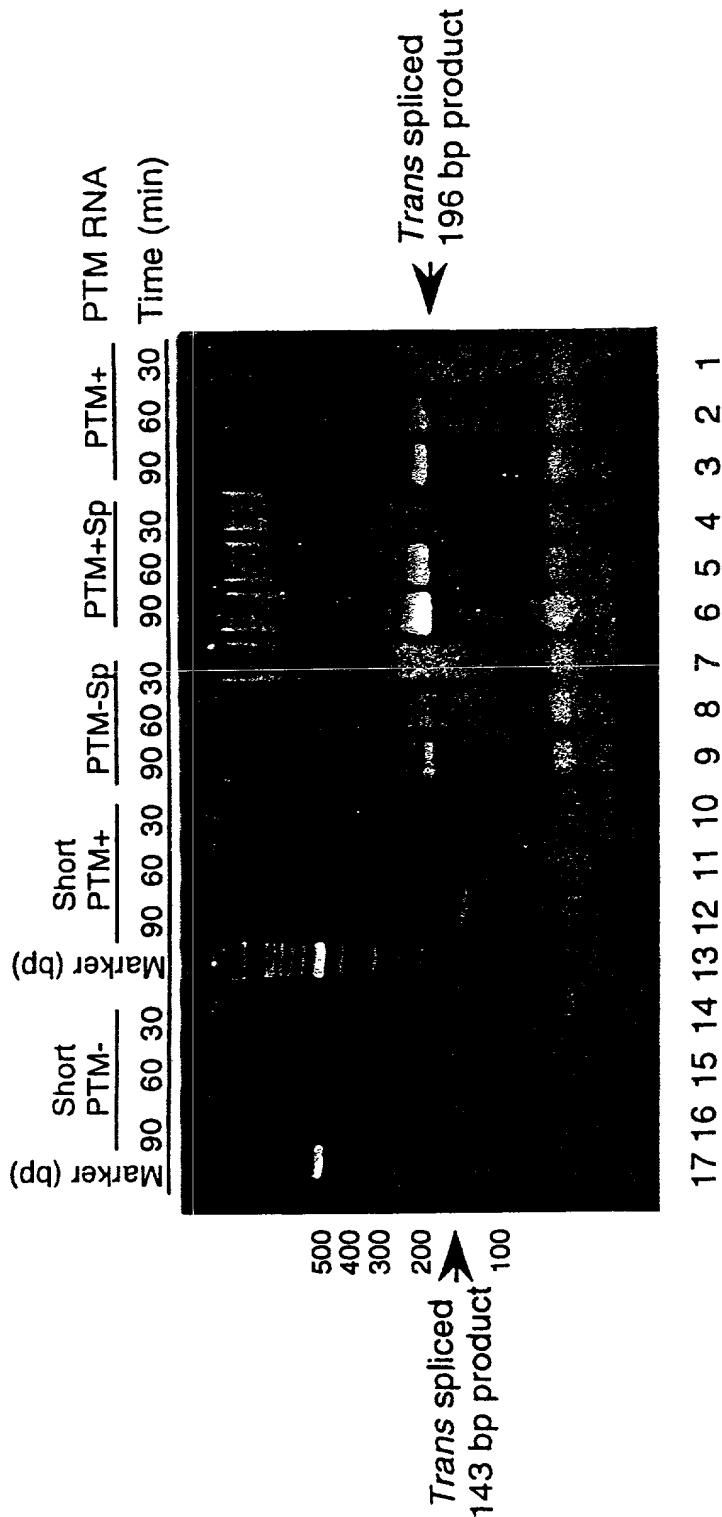


FIG.2B

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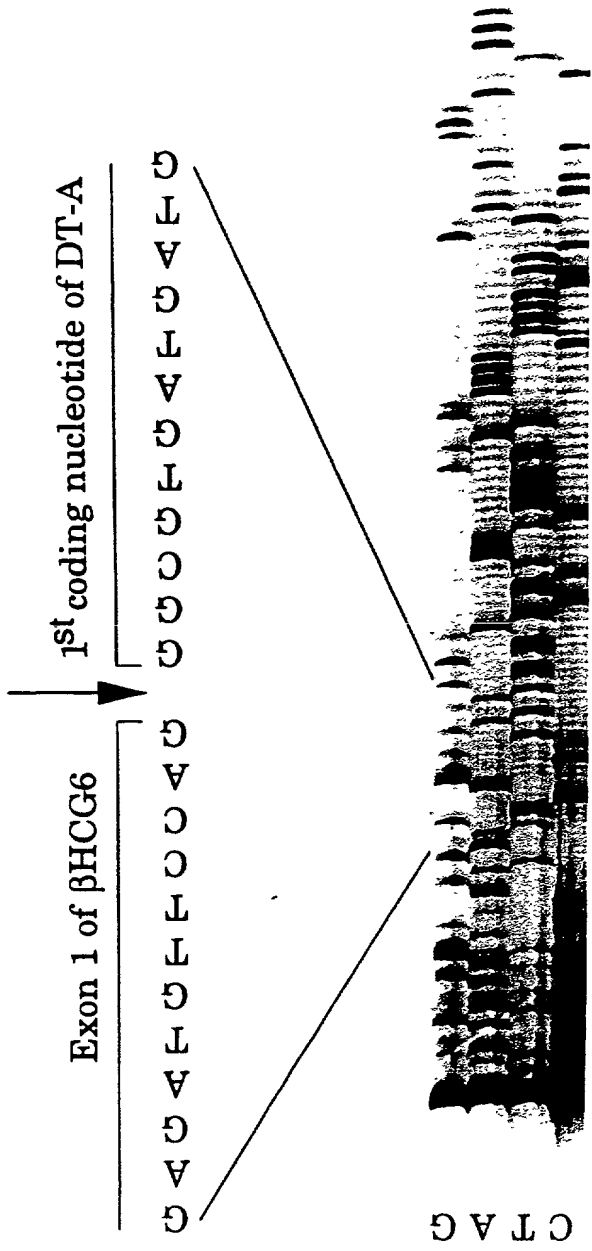
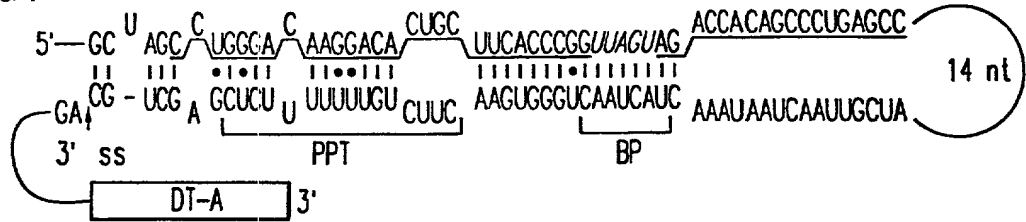
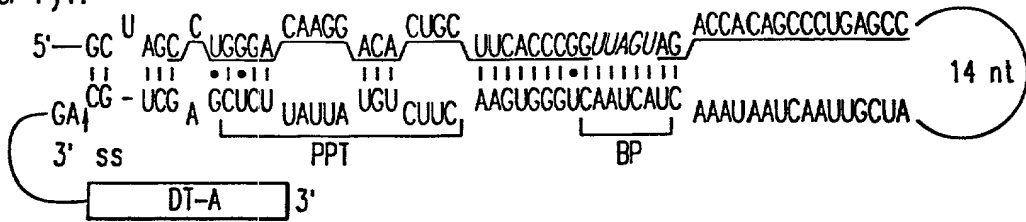


FIG. 3

1. PTM+SF:



2. PTM+SF-Py1:



3. PTM+SF-Py2:

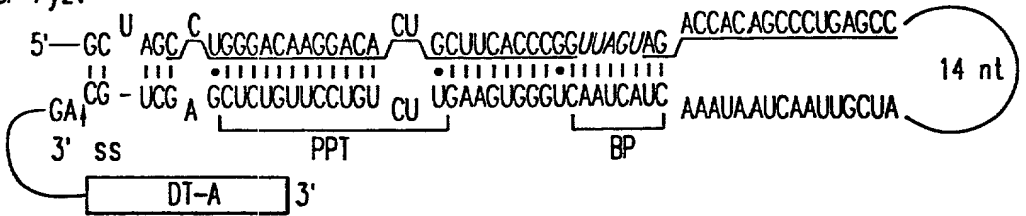


FIG.4A

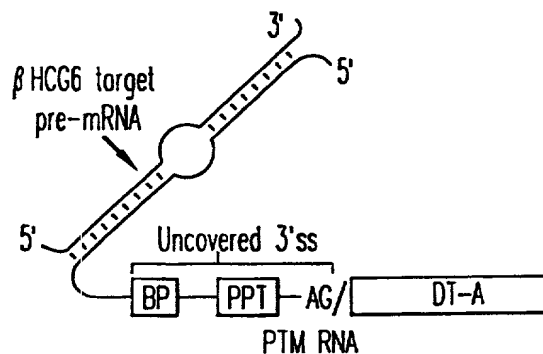


FIG.4B

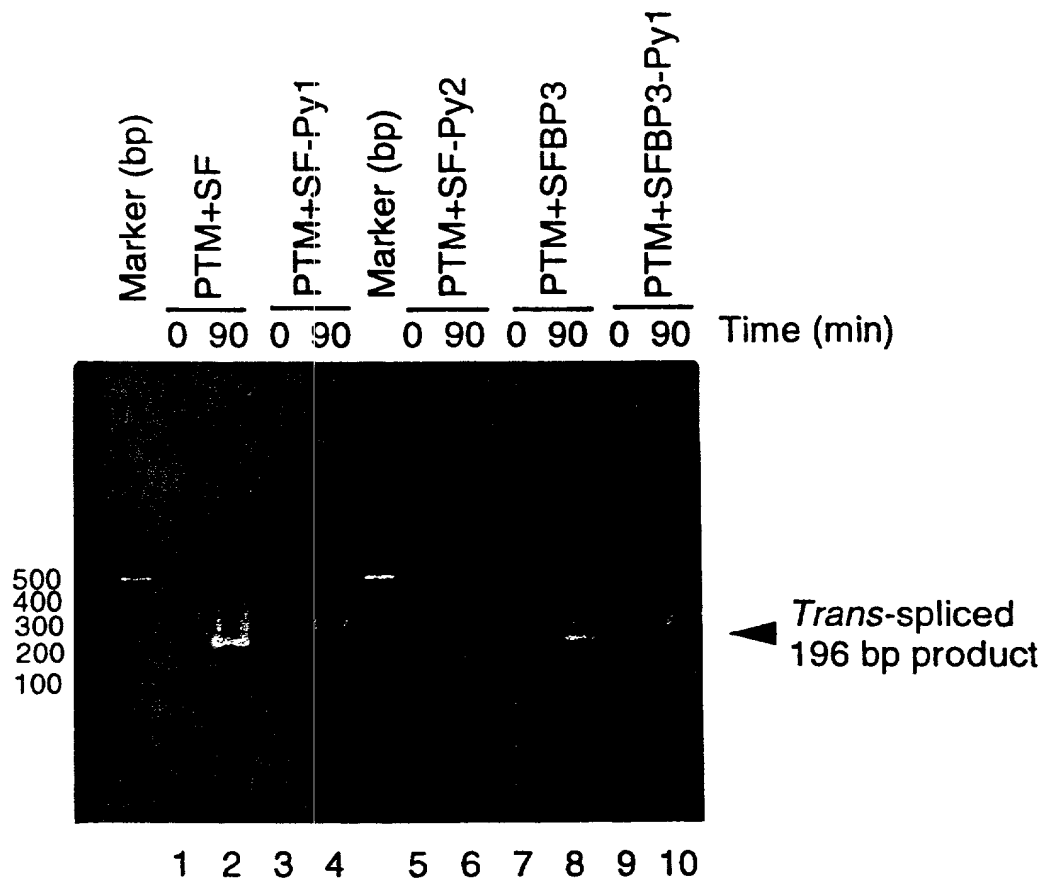


FIG.4C

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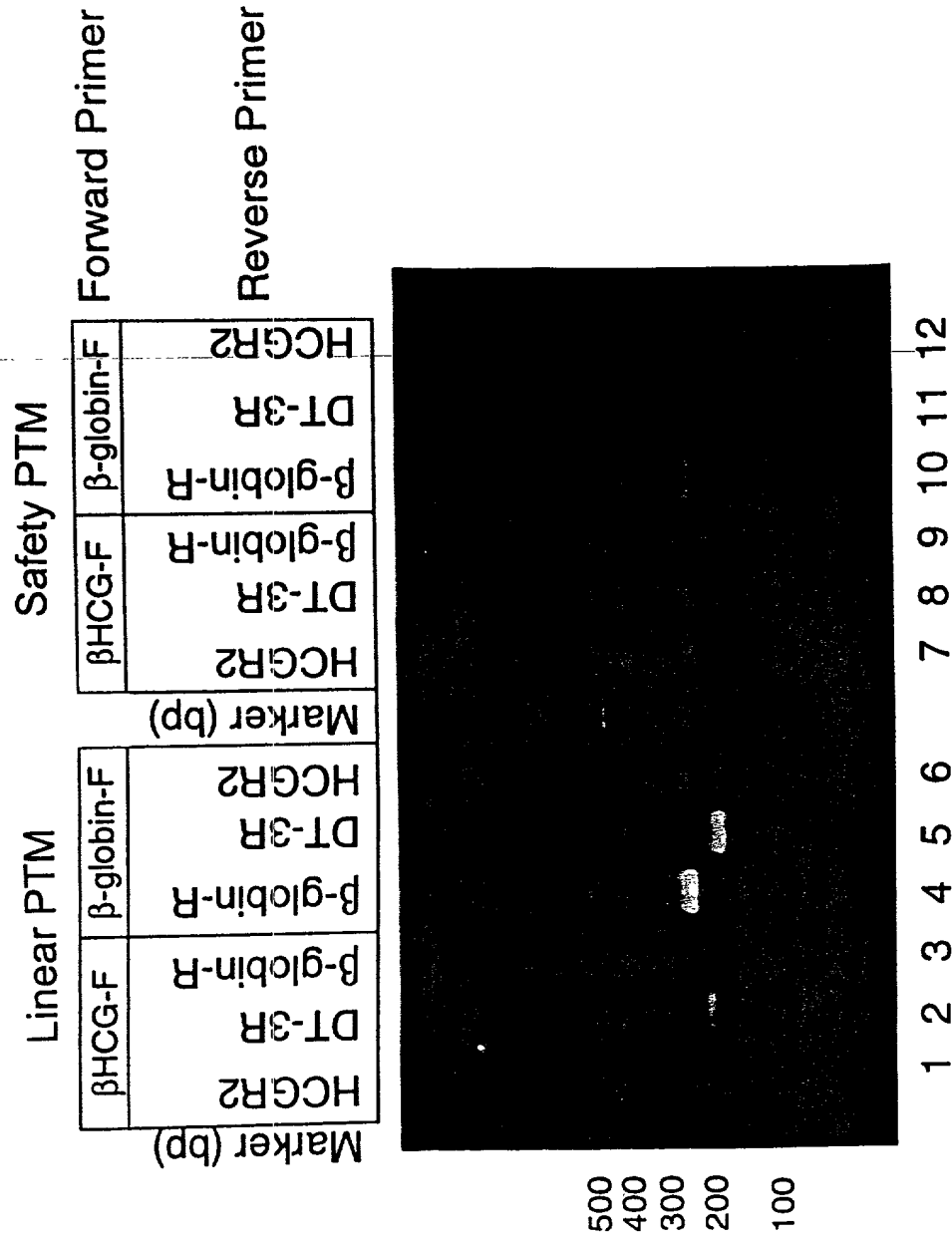


FIG.5

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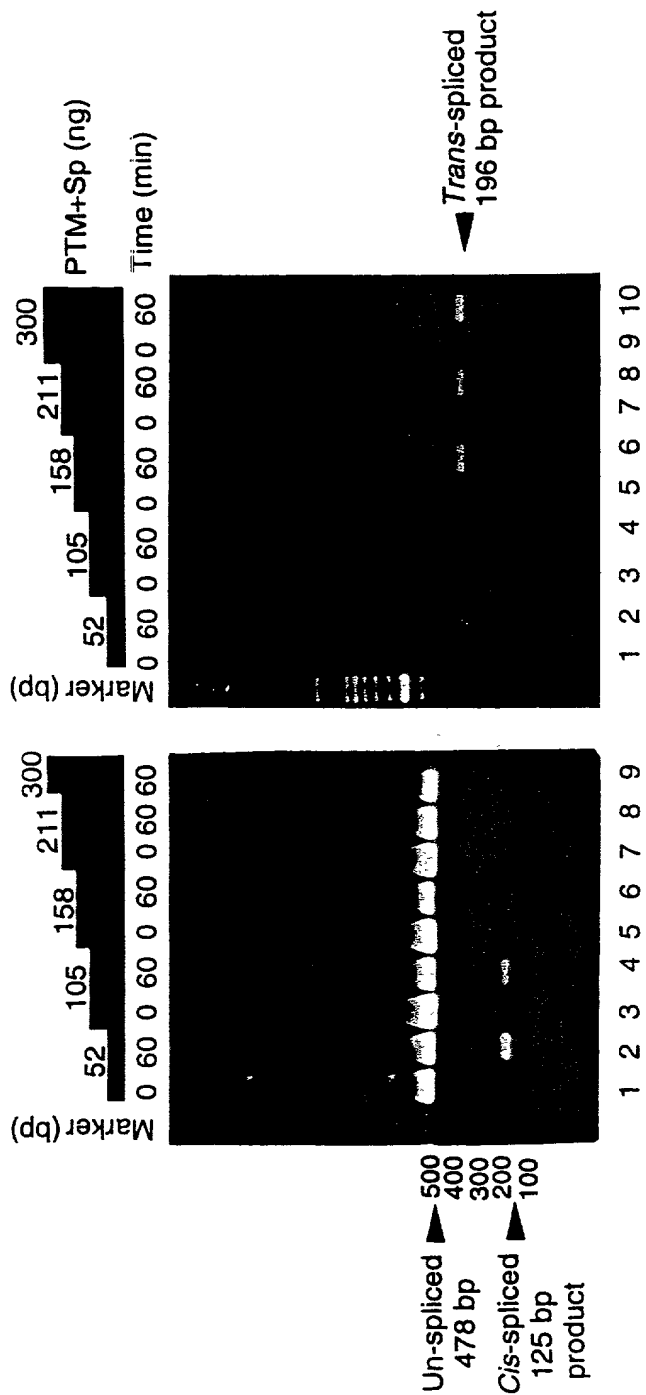


FIG. 6A

FIG. 6B

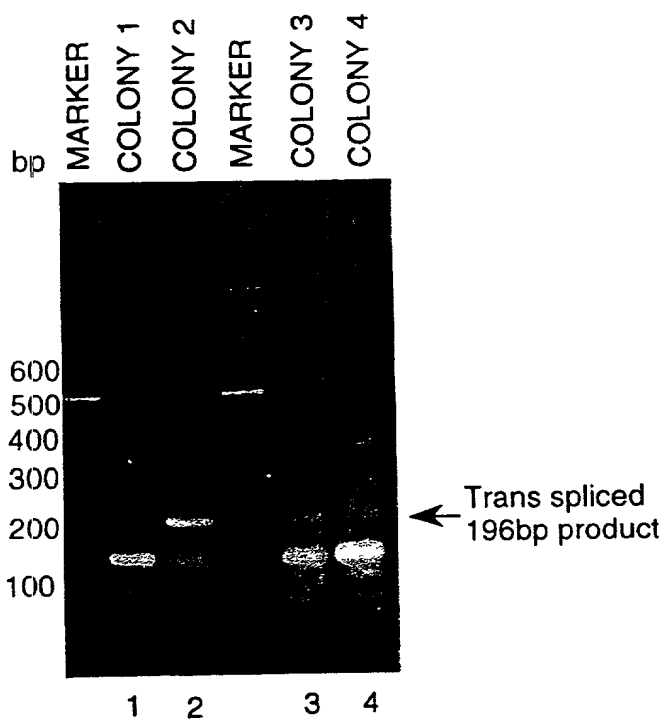


FIG.7A

68 *2* 11

EXON 1 OF β HCG6 ↓
 5'-CAGGGACGCCACCAAGGATGGAGATGTTCCAG-GCGCTGATGATGTTGTT
 ↓ 1ST CODING NUCLEOTIDE OF DT-A
 GATTCTTCTTAAATCTTTTGATGCGAAACCTTTCTTCGTACCAACGGGACTA
 AACCTGGTTAIGTAGATTCCATTCAAAAA-3'

FIG.7B

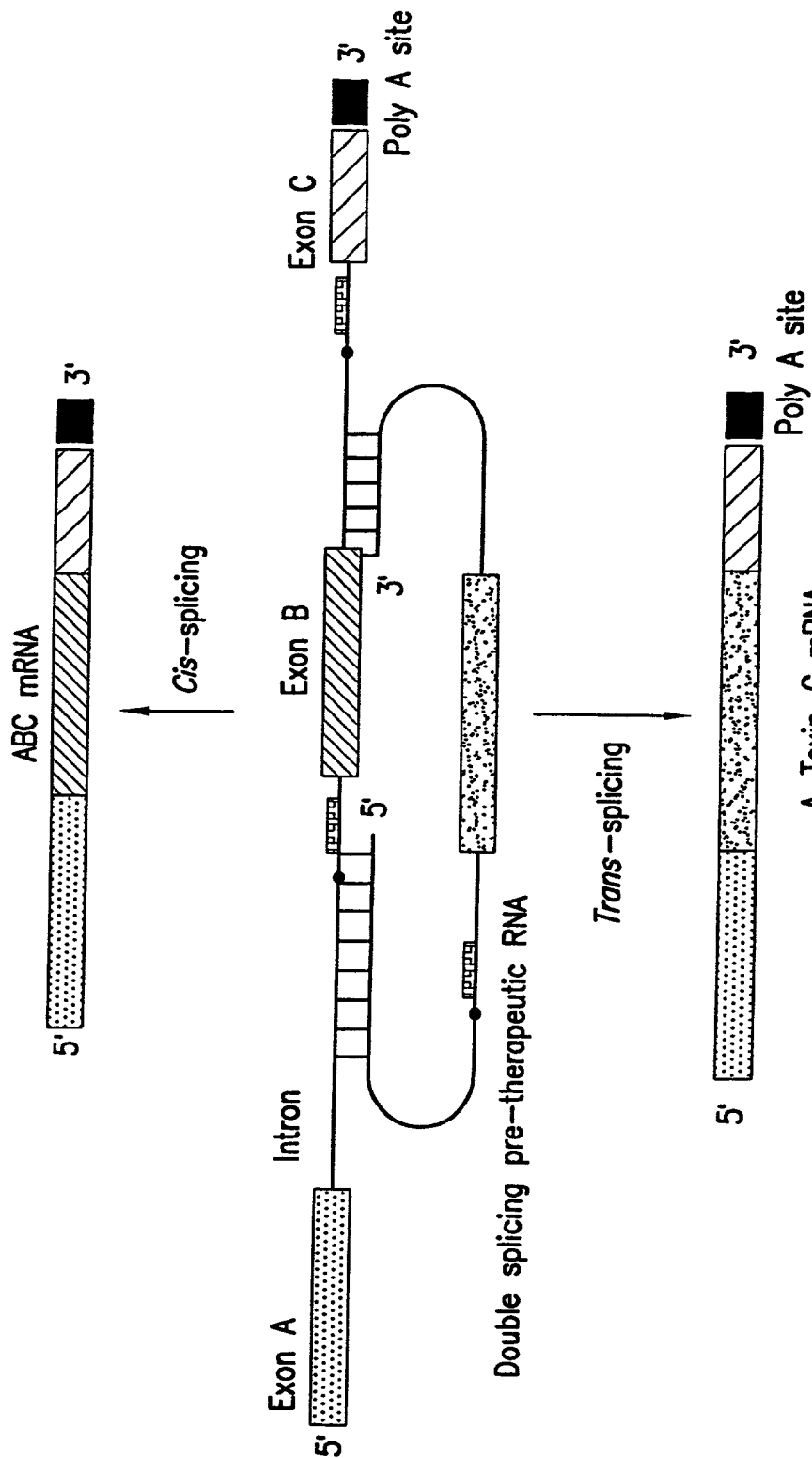


FIG.8A

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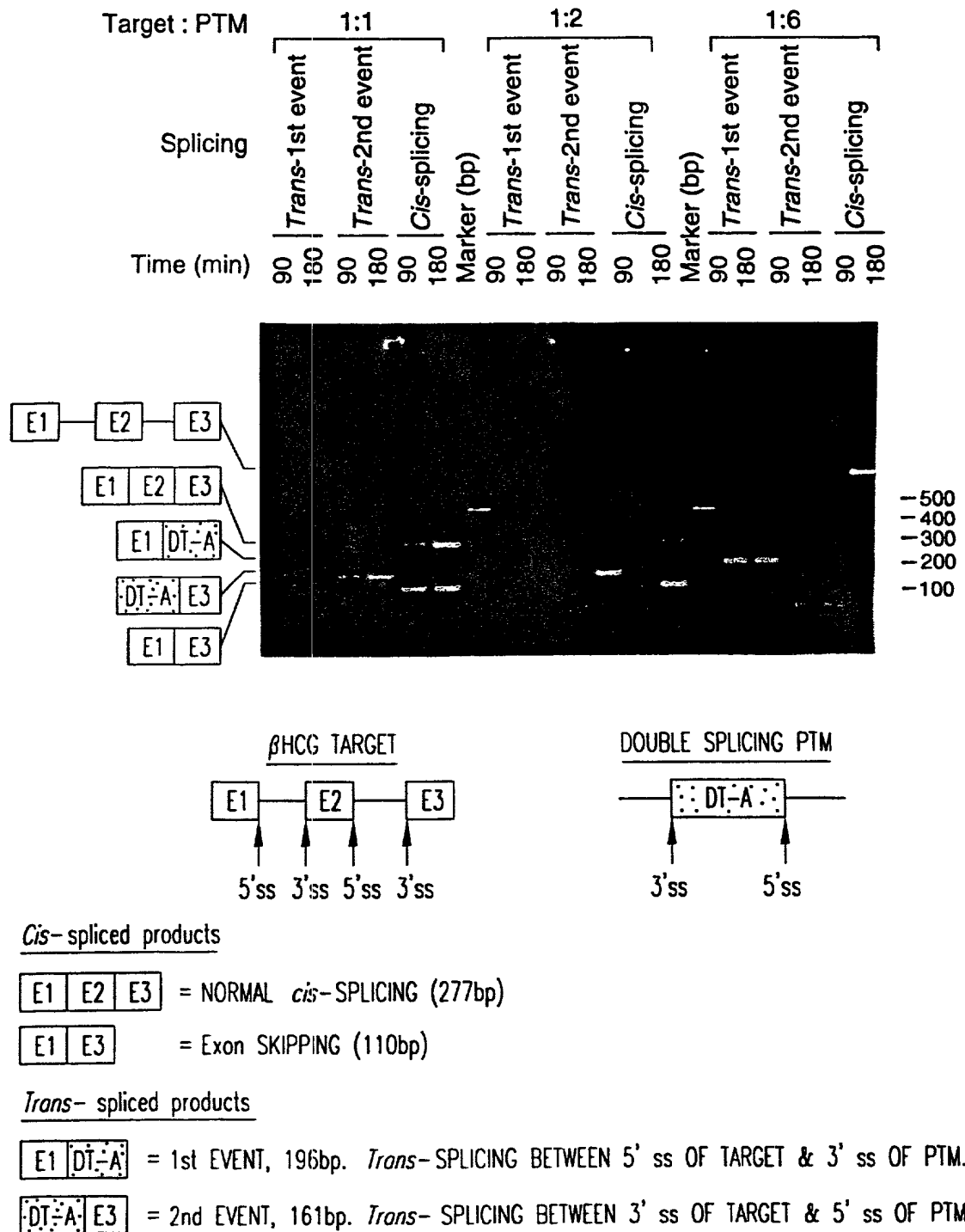


FIG.8B

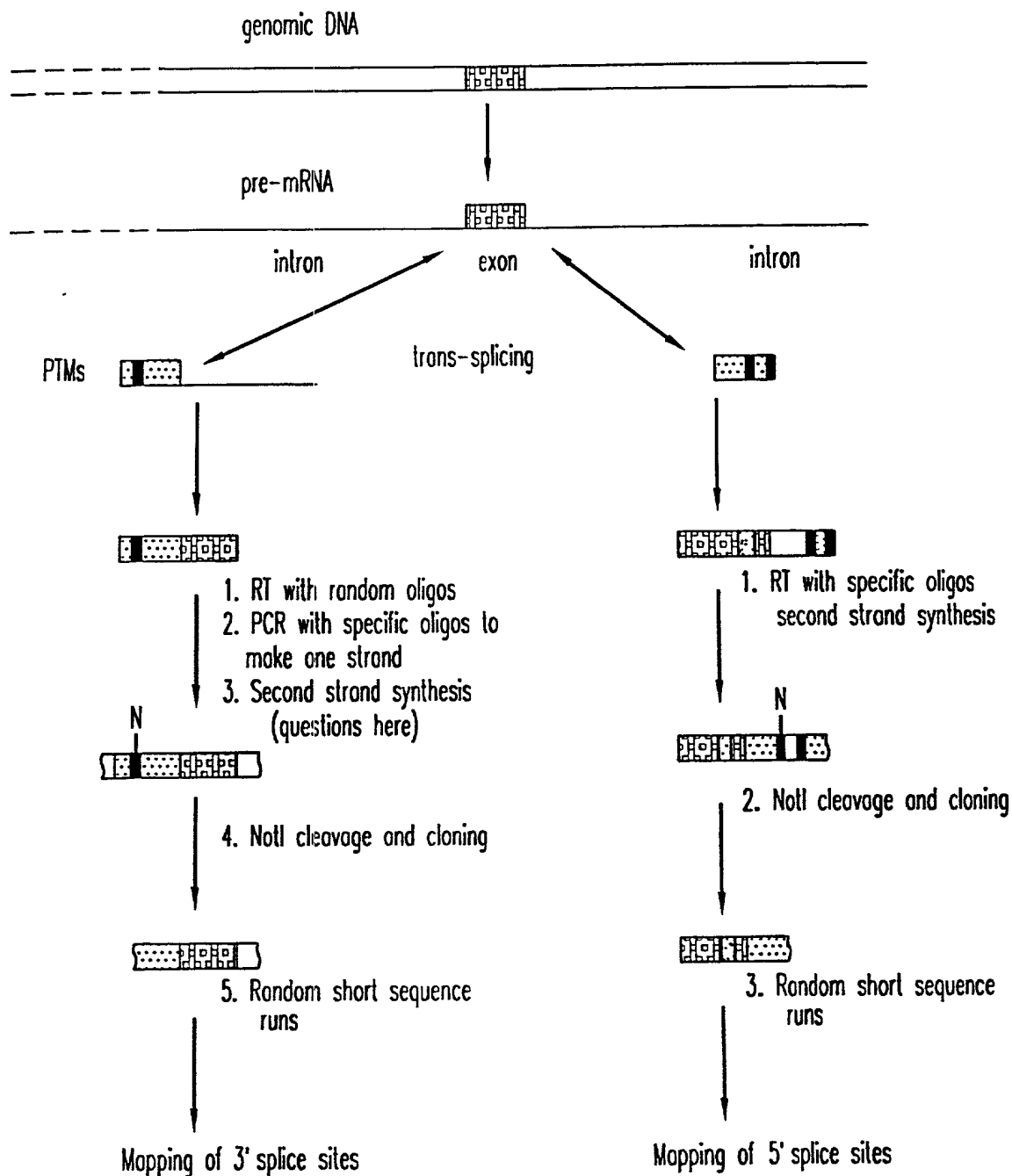
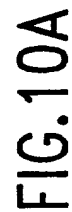


FIG.9



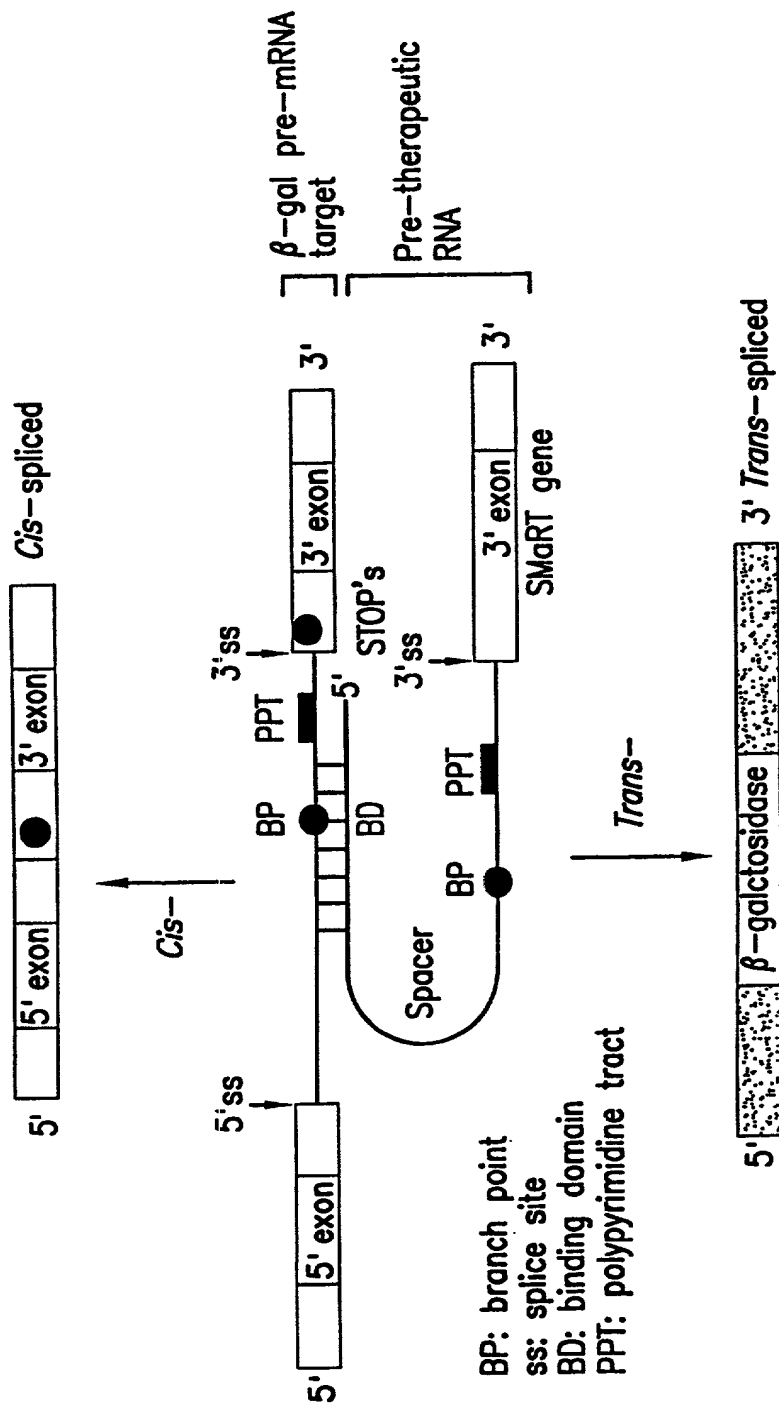


FIG.10B

68 21

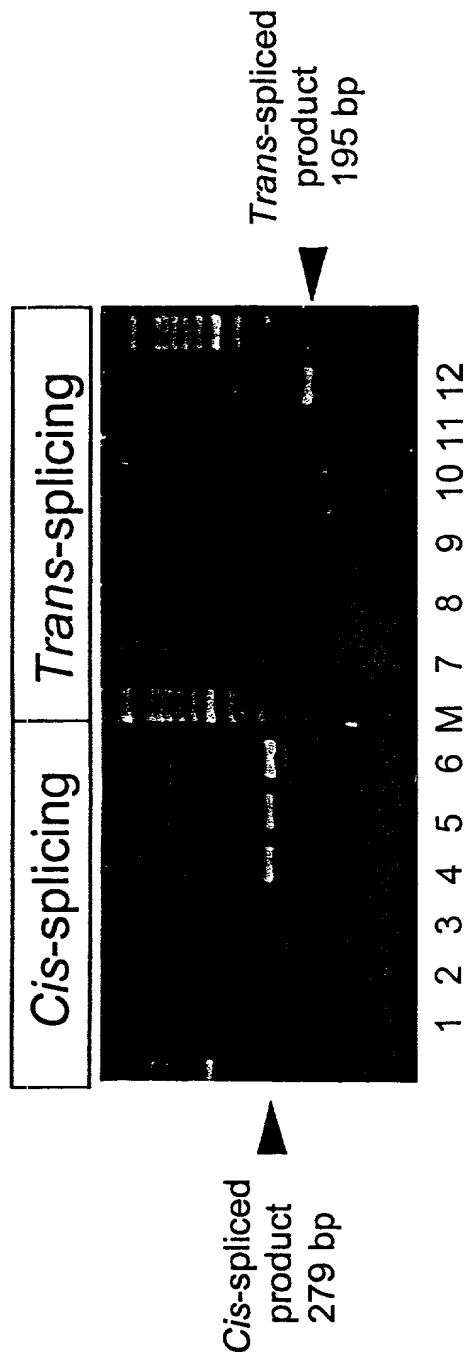
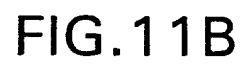


FIG. 11A

[illegible]

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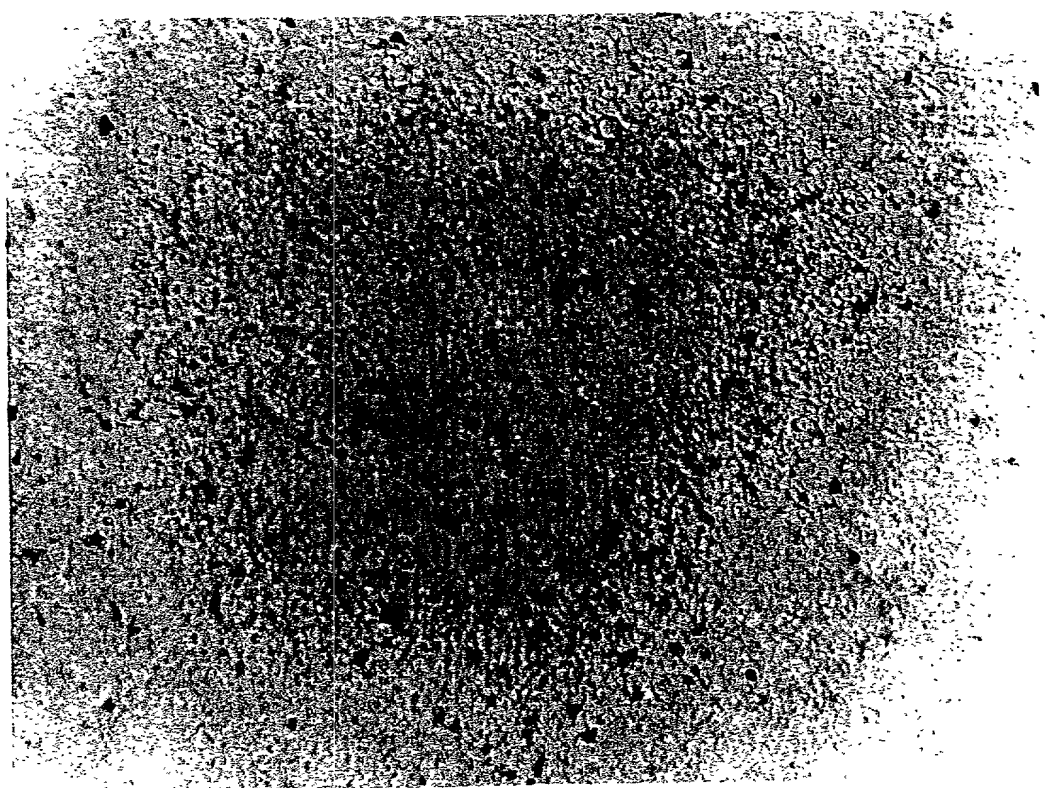


FIG.11C

09941492.082901

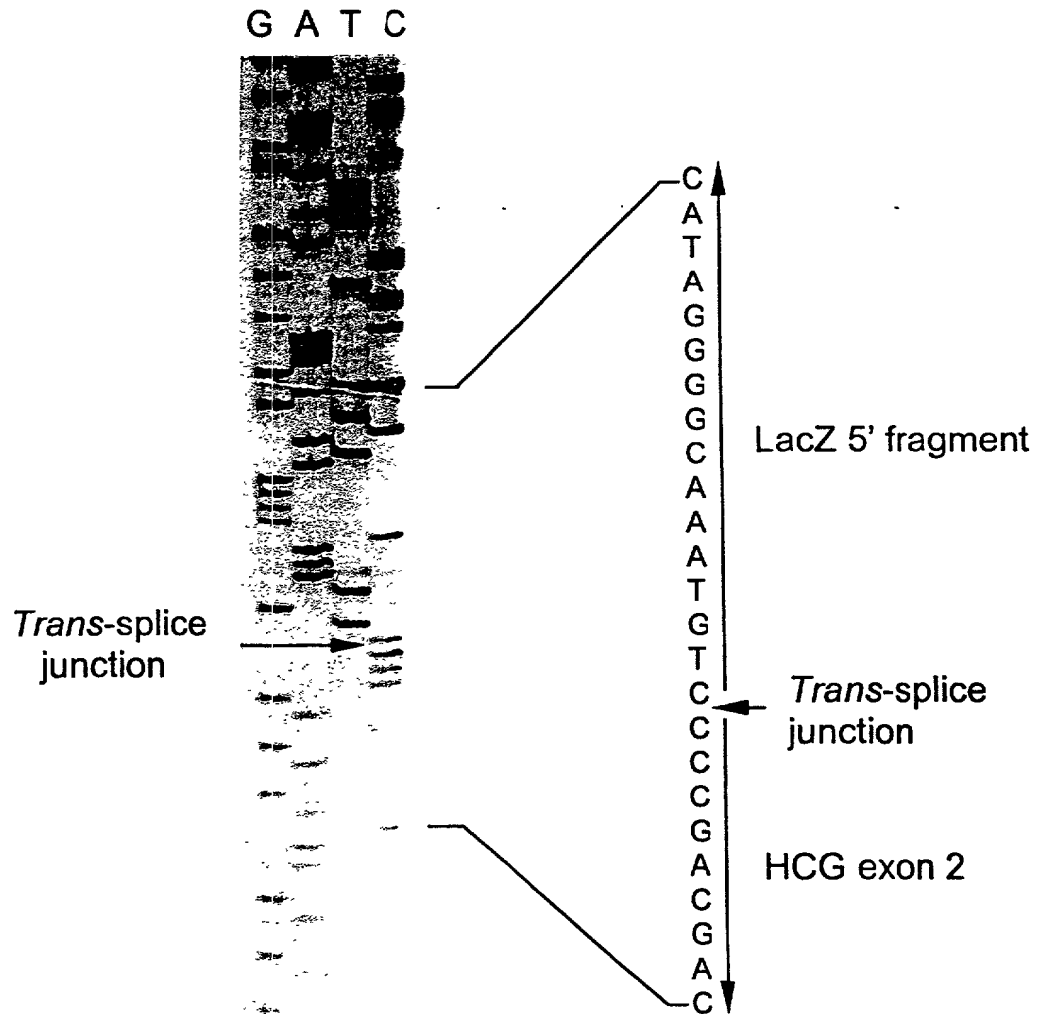
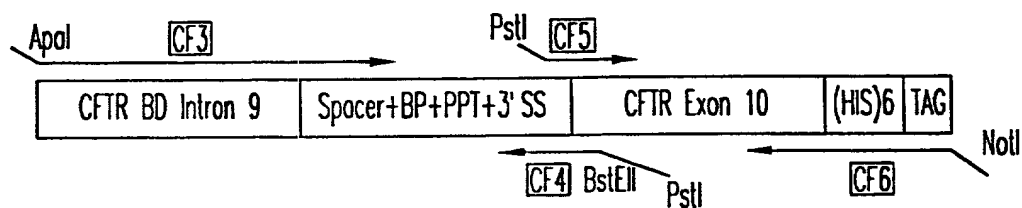
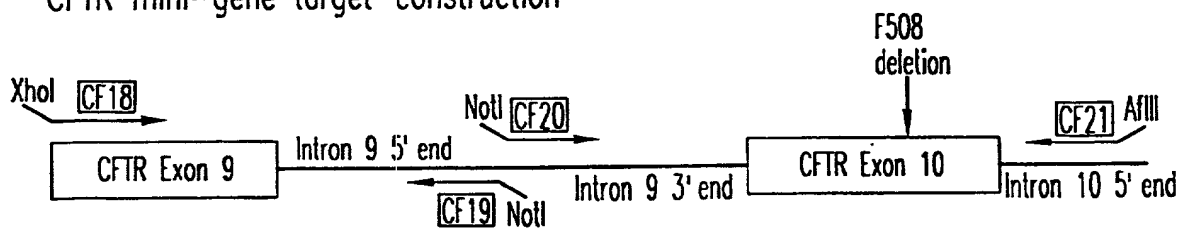


FIG.12A

CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target-construction



Trans-splicing Repair

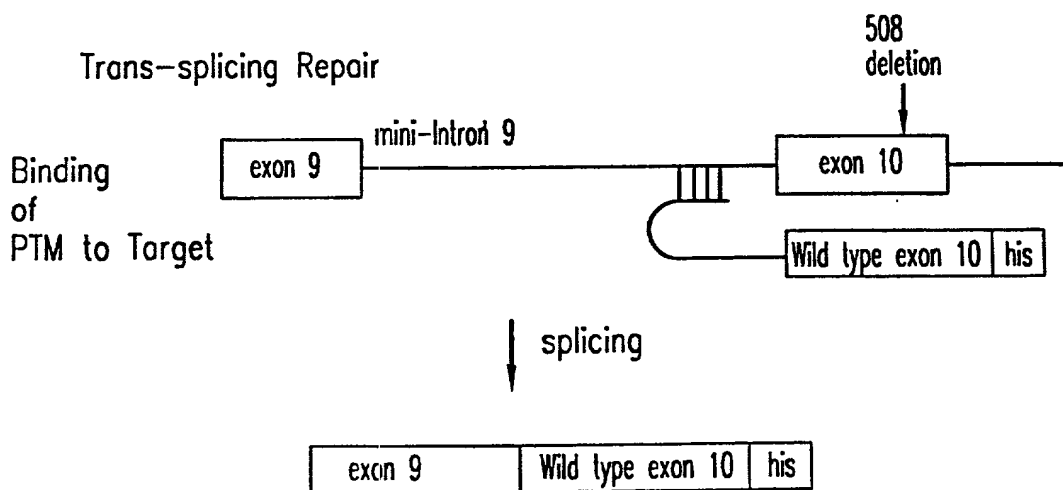


FIG.13

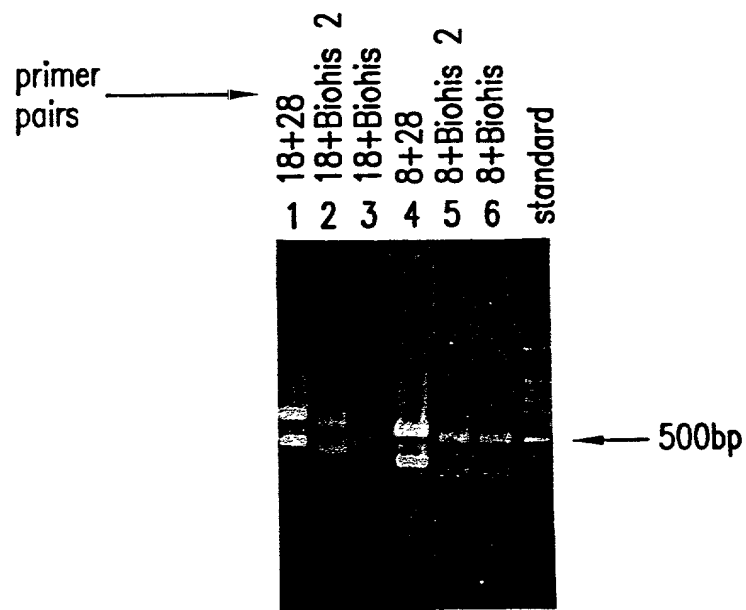
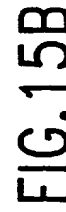


FIG.14

Positions of Restriction Endonucleases sites (unique sites underlined)

Kpn I Pst I 3'ss
 ↓ ↓ ↓
 TGGTACCTCTTCTTTTTTTCCTGGCAGACTTCACCTTCTAATGATGATTATGGGAGAACTGGAGCCCTTCAGAGGGTAAAT
 ACCATGGAGAAGAAAAAAGGACGCTCGAAGTCAAGATTACTACTAATACCCCTCTTGACCTCGGAAGTCTCCCATTTTA
 82 102 160
 Xmn I Dde I F508
 ↓ ↓ ↓
 TAAGCACAGTGGGAAGAAATTCATTCTGTTCTCAGTTTTCTCGGATTATGCCTGGCACCATTAAAGAAAAATATCATCTTGG
 ATTCGTGTCACCTTCTTAAAGTAAGACAAGAGTCAAAAGGACCTAATACGGACCGTGGTAATTTCTTTTATAGTAGAAAC
 172 190 240
 Sph I HIS STOP
 ↓ ↓ ↓
 GTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAAGCATGCCAACTAGAAGAGCATCATCATCATCATATAG
 CACAAAGGATACTACTTATATCTATGCTTCGCAGTAGTTTCGTAACGGTTGATCTTCTCCGCTAGTAGTAGTAGTAATC
 282 320

FIG. 15A





+

F508 deletion

CFTR Target
(mini-gene)



Mini-intron 9
(~0.6kb)

Cotransfect PTM and target molecules in HEK 293 cells
and detect repaired CFTR mRNA by RT-PCR.

Repaired
CFTR mRNA

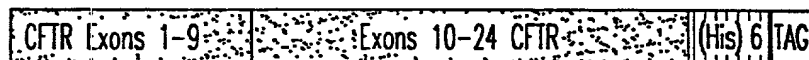


FIG.16

Double Splicing
PTM

CFTR BD intron 9	Spacer+BP+PPT+3'SS	CFTR exon 10	Spacer+BP+PPT+5'SS	CFTR BD intron 10
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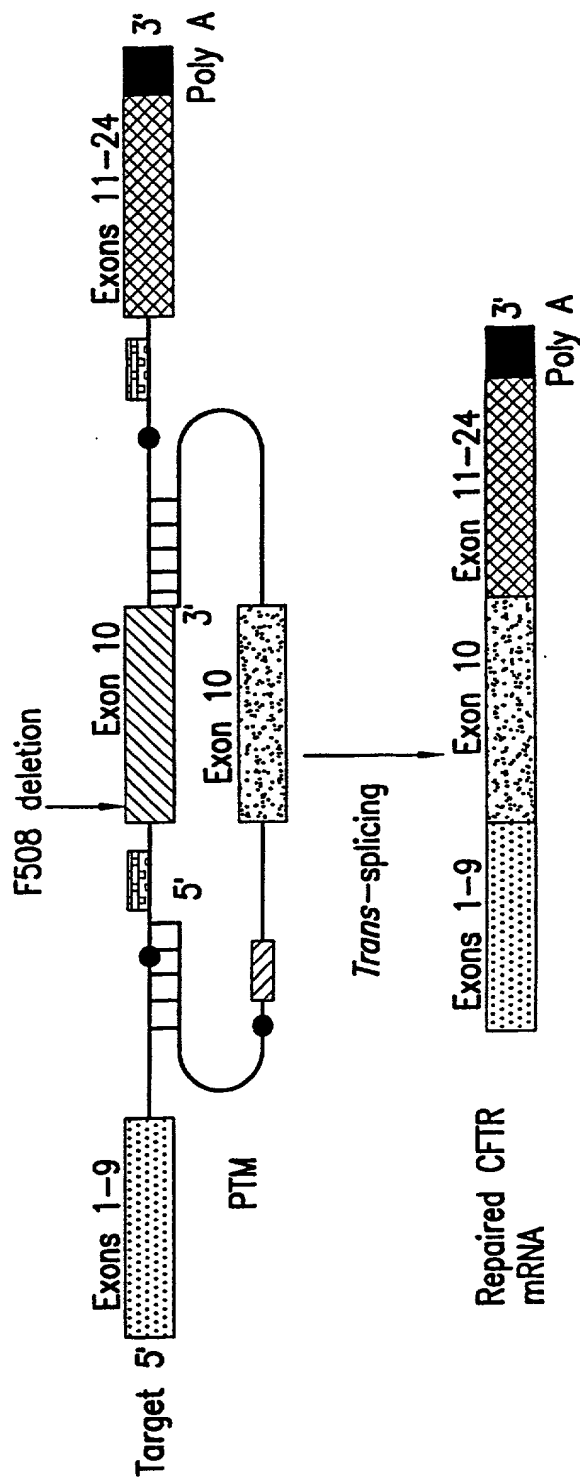


FIG.17

Double Trans-splicing Specific Target

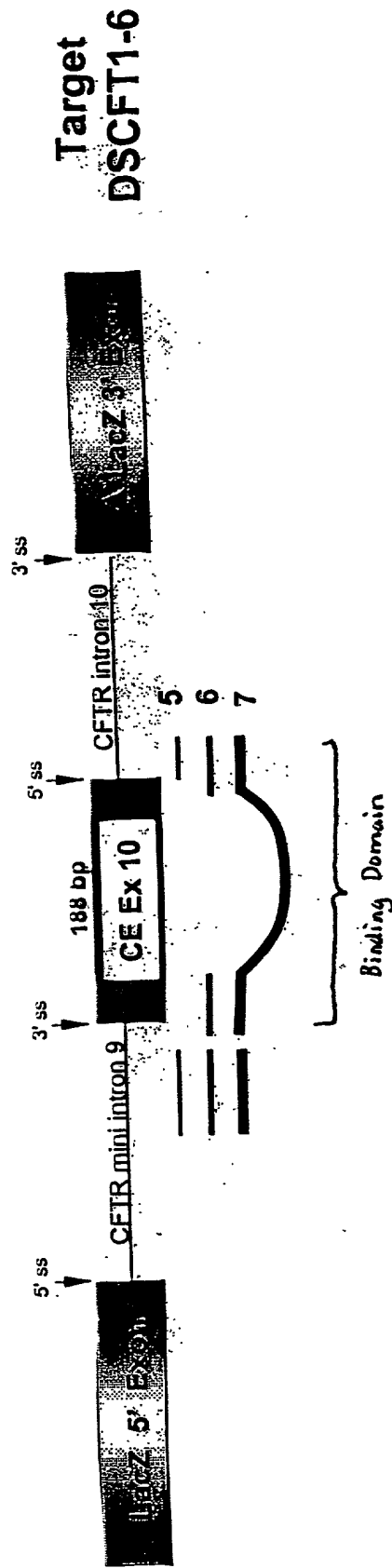


Figure 18

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Double Splicing PTMs

Double Trans-splicing PTMs

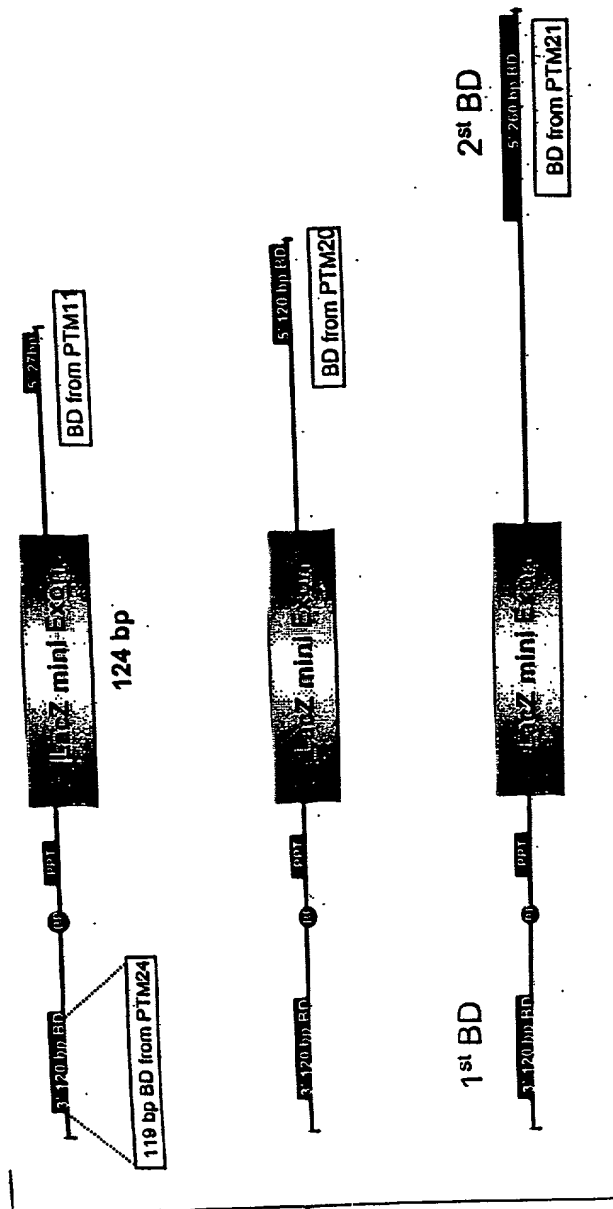


Figure 19

Double Trans-splicing β -Gal Model

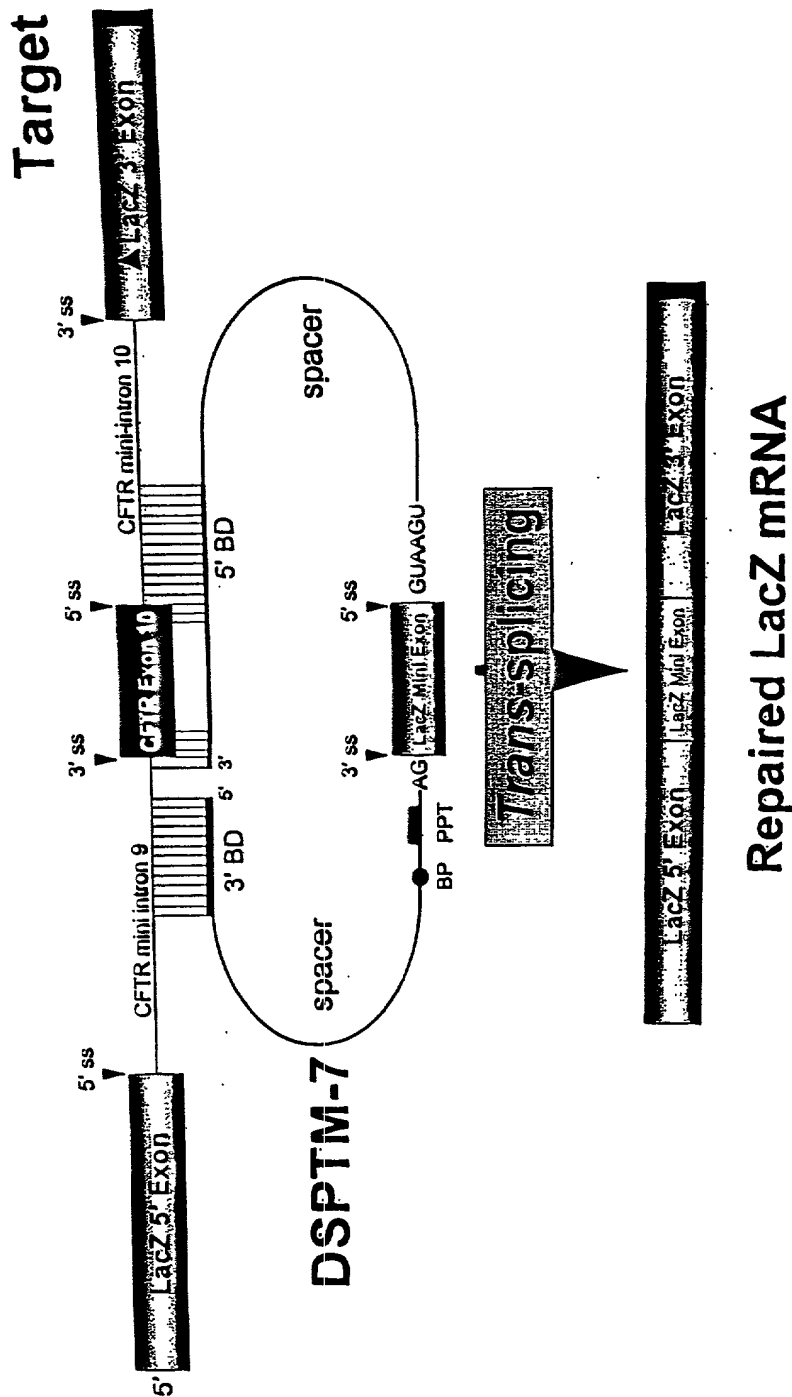
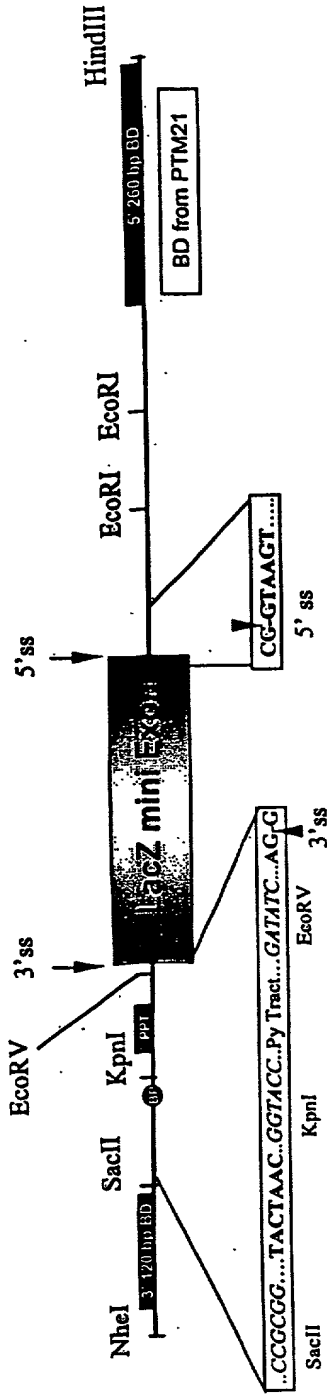


Figure 20

Important Structural Elements of DSPTM-7: (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



(1) 3' BD (120 BP) : GATTCACCTTGCTCCAAATTATCATCTCAAGCAGAGTGATATCTTATTGTAAAGATTCTTAACTCATTTGATTC
AAATATTTAAATACTCTCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

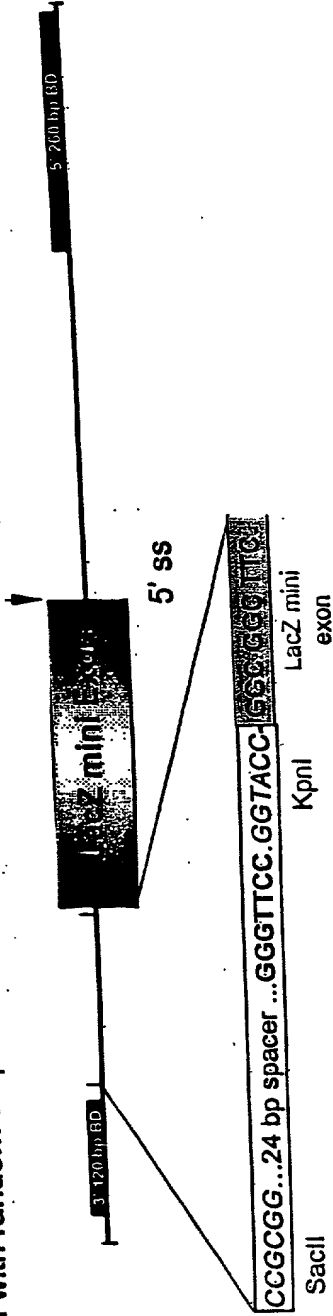
(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T GGTACC TCTTCTTTTTTTTTT GATA TC CTGCAG **LacZ mini exon**
BP Kpn I PPT EcoRV 3' ss

(4) 5' donor site and 2nd spacer sequence: **LacZ mini exon** GTAAGT GTTATCACCAGATATGTCTAACCTGATTCTGGGCTTCGATACG
CTAAGATCCACCGG

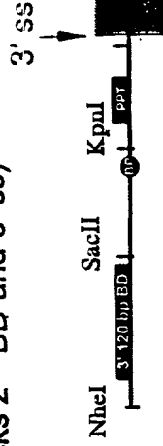
(5) 5' BD (260 BP) : TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTTGAAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATTCATTGGAA
ACACCAATGATTTTCTTTAATGGTGCCTGGCATAATCCTGGAAACTGATAACACAAATGAAATTTCTTCCACTGTGCTTAA
AAAAACCTCTGAAATTCCTCAATTTCTCCCATATATCATCATTAACAACTGAACCTCTGGAAATAAAACCCATCATTTAACTCA
TTATCAAAATCACGC

Figure 21

DSPTM8 : (▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)



PTM29 (lacks 2nd BD and 5' ss)



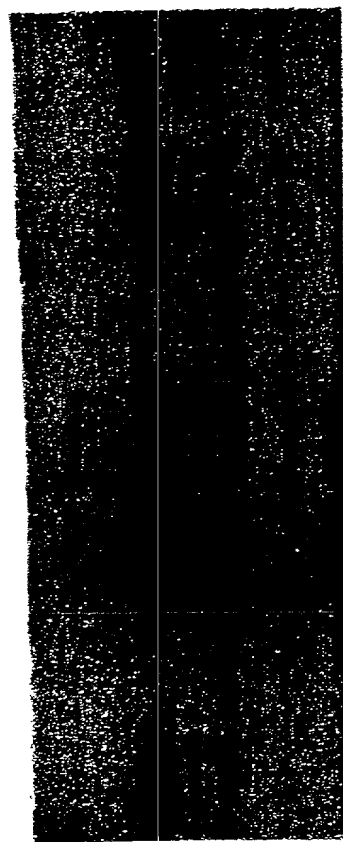
PTM30 (lacks 1st BD and 3' ss)



Figure 22

Mutants

Double Trans-splicing Produces Full-length Protein



β-gal →
(120 kDa)

1 2 3 4 5 6 7

Lane 1: DSCFT1.6 Target alone 25 μg
 Lane 2: DSPTM7 25 μg
 Lane 3 Target + PTM #6 25 μg
 Lane 4: Target + PTM #9 25 μg
 Lane 5: Delta 3' splice mutant alone 25 μg
 Lane 6: Target + Delta 3' ss 25 μg
 Lane 7: Target+PTM29+30 (mutants) 25 μg

Figure 24

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Restoration of β -Gal Function by Double Trans-splicing

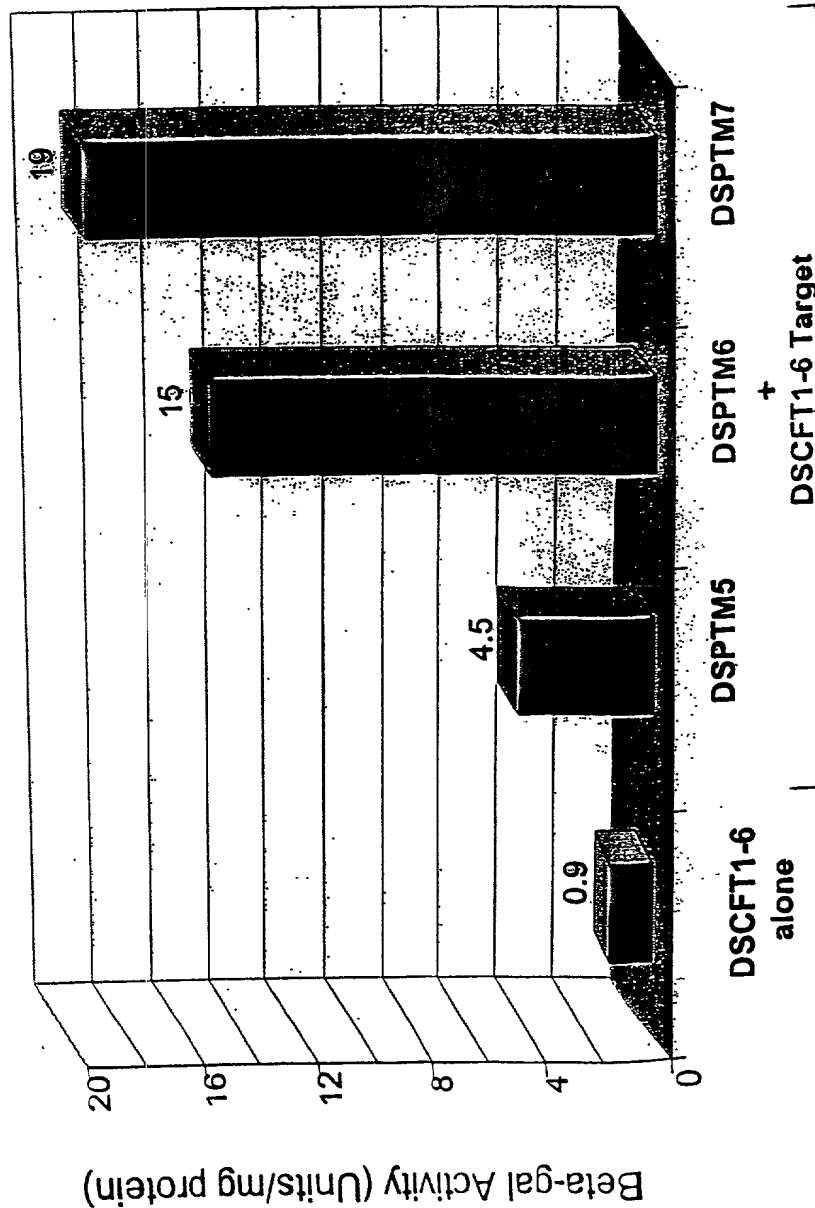


Figure 25

Restoration of β -gal activity is due to double RNA trans-splicing events

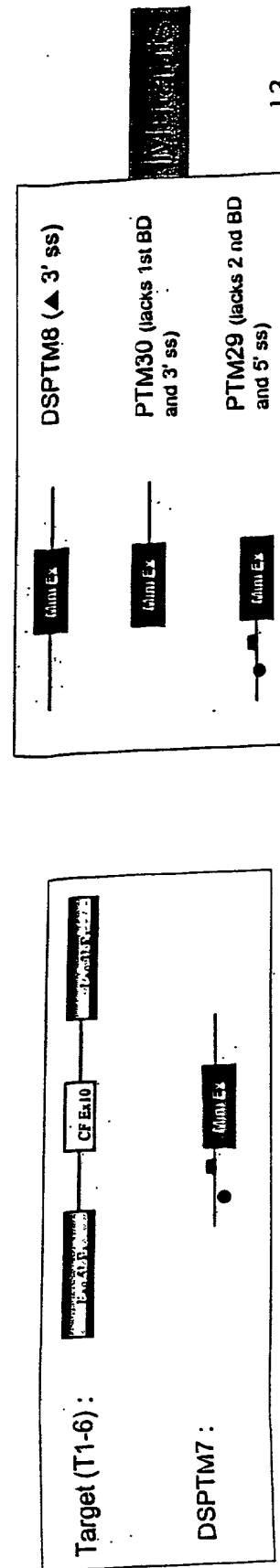
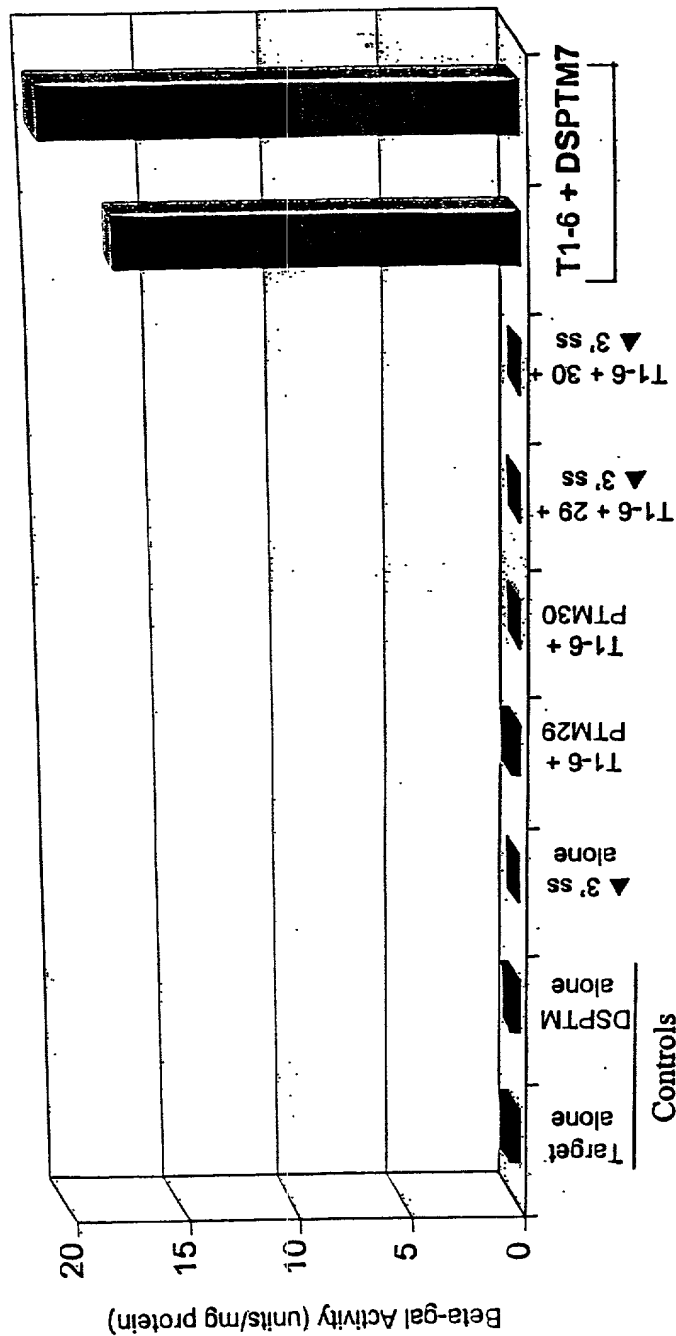


Figure 26

106230 2544650

Double Trans-splicing: Titration of Target & PTM

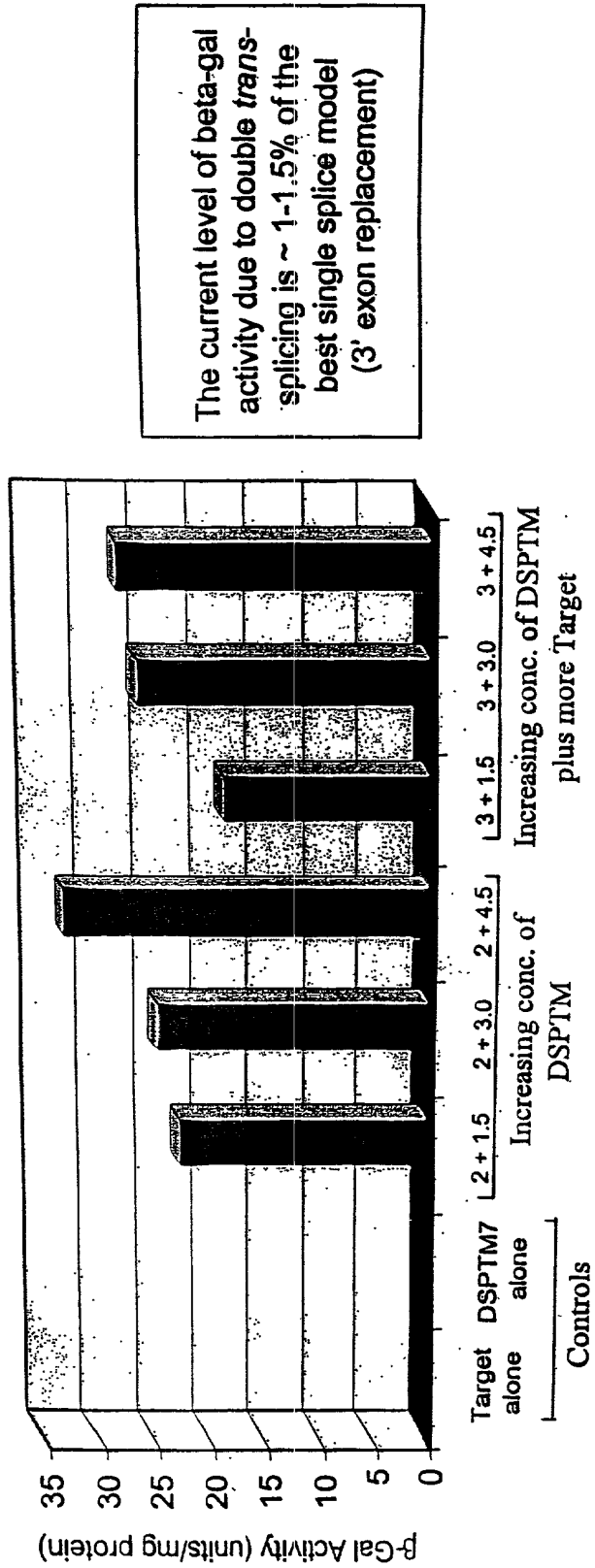
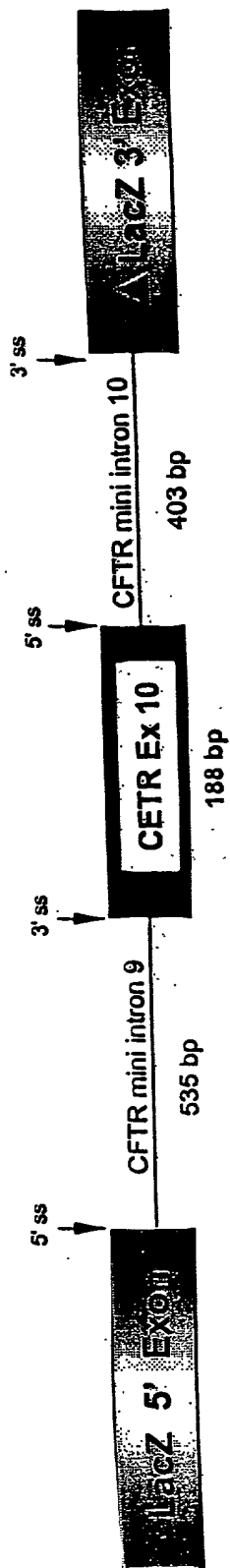


Figure 27

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DSCFT1-6 (Specific Target):



DSHCGT1 (Non-specific Target):

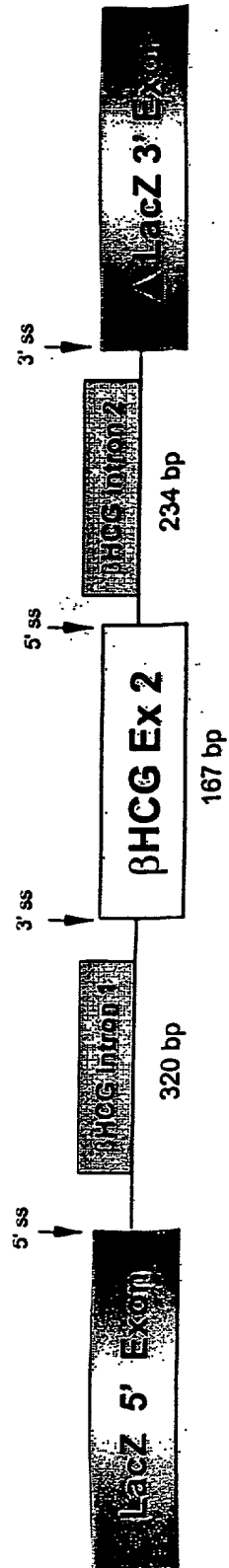


Figure 28

Specificity of double *trans*-splicing Reaction

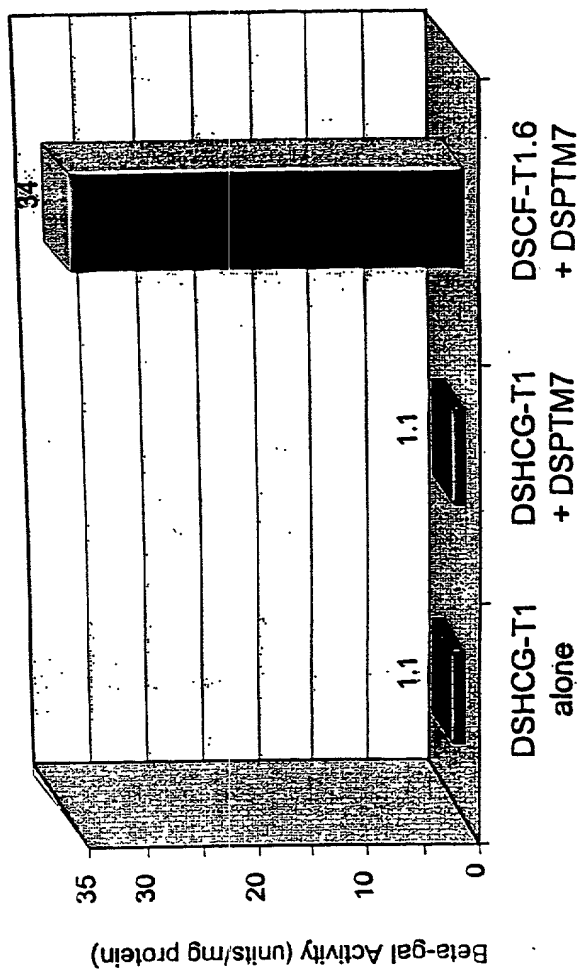


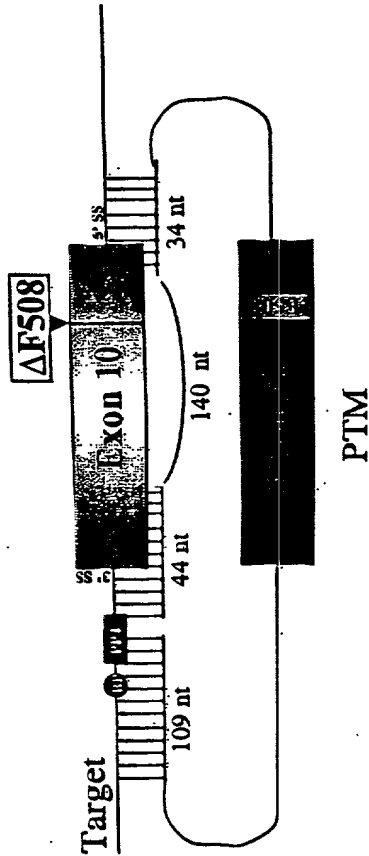
Figure 29

The diagram illustrates the targeting strategy for the CFTR gene. It shows the CFTR gene structure with exons 9, 10, and 11, and introns 9 and 10. A targeting vector (PTM) is shown with a poly(A) signal (Poly(A)) and a targeting site (BD) flanked by loxP sites (green triangles). The targeting process involves homologous recombination (indicated by arrows) to replace the wild-type exon 10 with the PTM. The resulting CFTR mRNA is shown with the PTM inserted into exon 10, flanked by loxP sites, and followed by a poly(A) signal. The final product is the repaired full-length CFTR mRNA.

Figure 30

NITRON

PTM with a long binding domain masking
two splice sites and part of exon 10
in a mini-gene target.



ACGAGCTTGCTCATGATCATGGCGAGTTAGAACCAAGTGAAGGCAAGATCAACAATTCCG
GCCGCATCAGCTTTTGCAGCCAATTTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCCTGTCAGTTGGAGGAG

MCU in exon 10 of PTM

88 of 192 (46%) bases in PTM exon 10 are not complementary to
its binding domain (bold and underlined).

Figure 31

INTRONIN

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Sequence of a double
trans-spliced product

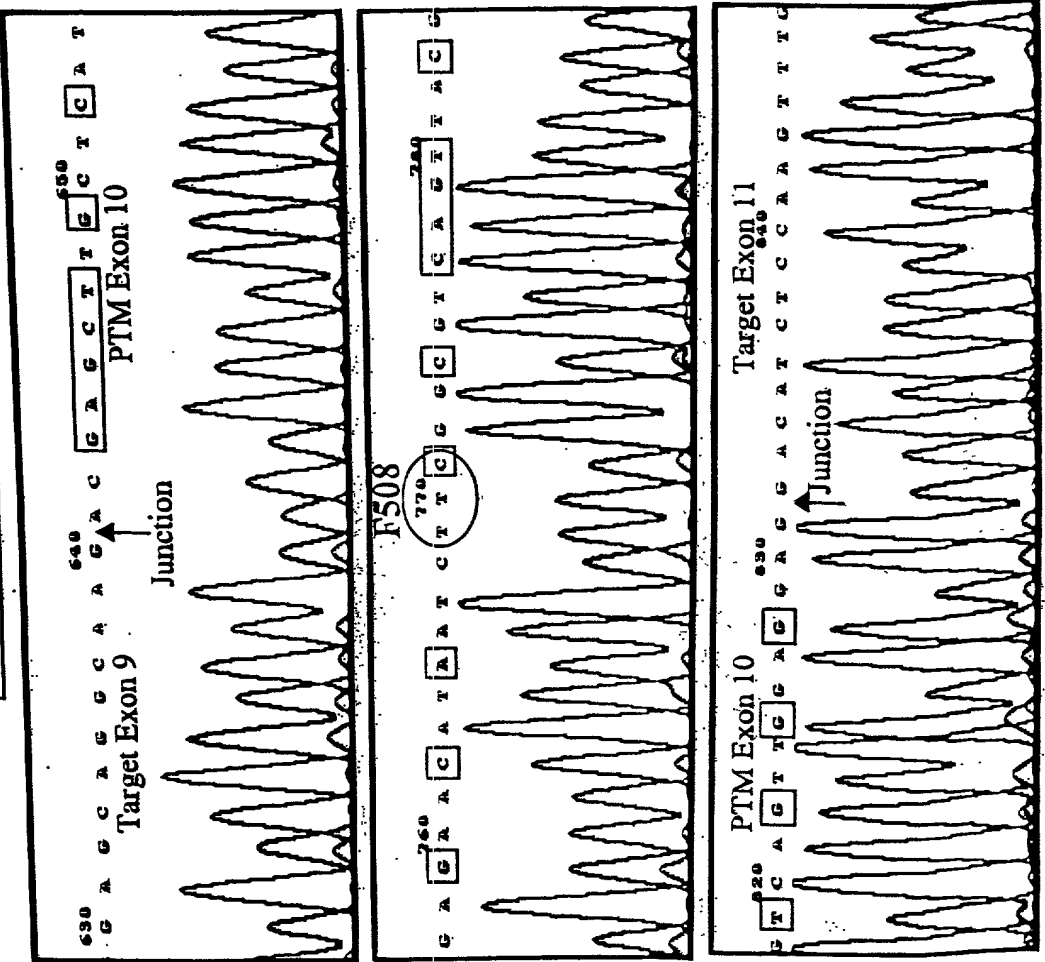


Figure 32

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CFTR Repair: 5' Exon Replacement

Schematic diagram of a PTM binding to the splice site of intron 10 of a mini-gene target.

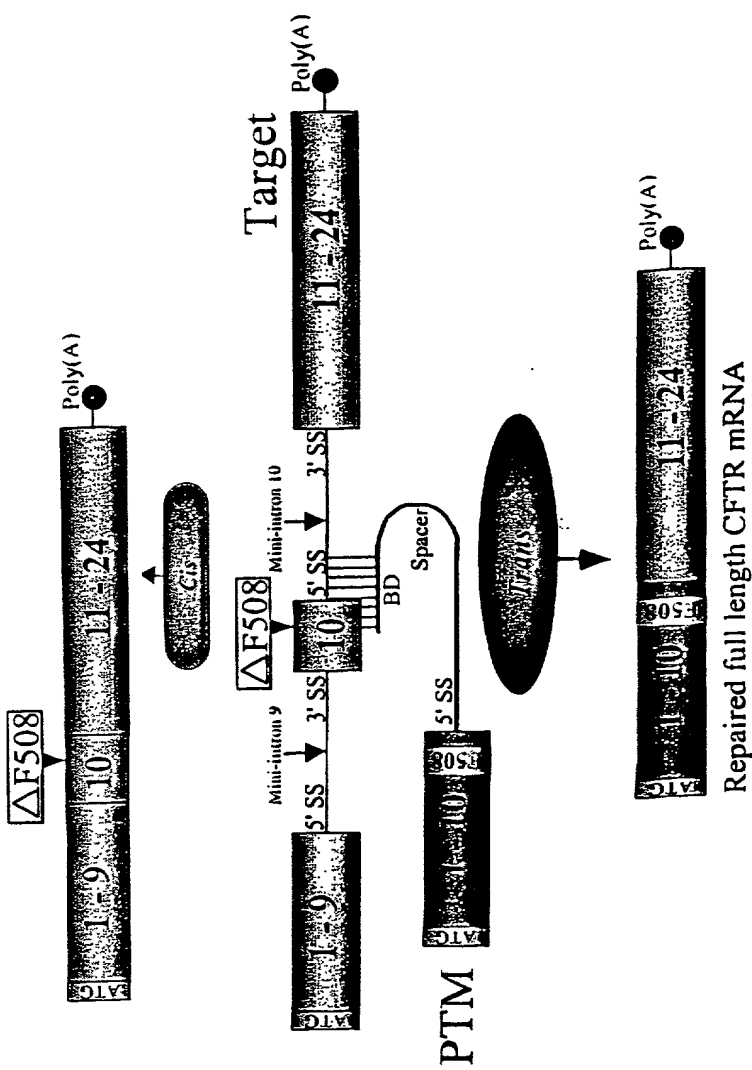
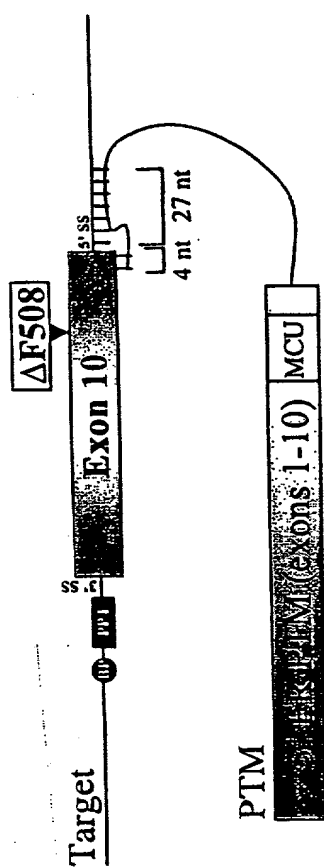
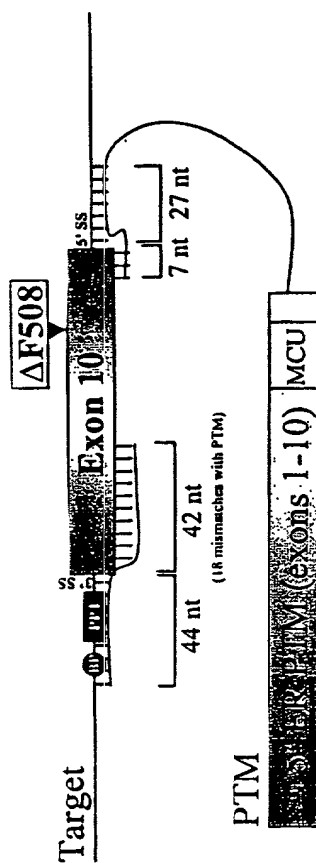


Figure 33

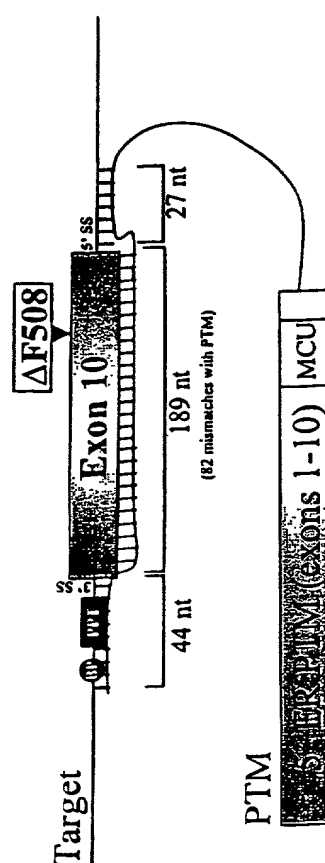
1110000



PTM with a short binding domain masking a single splice site in a mini-gene target.

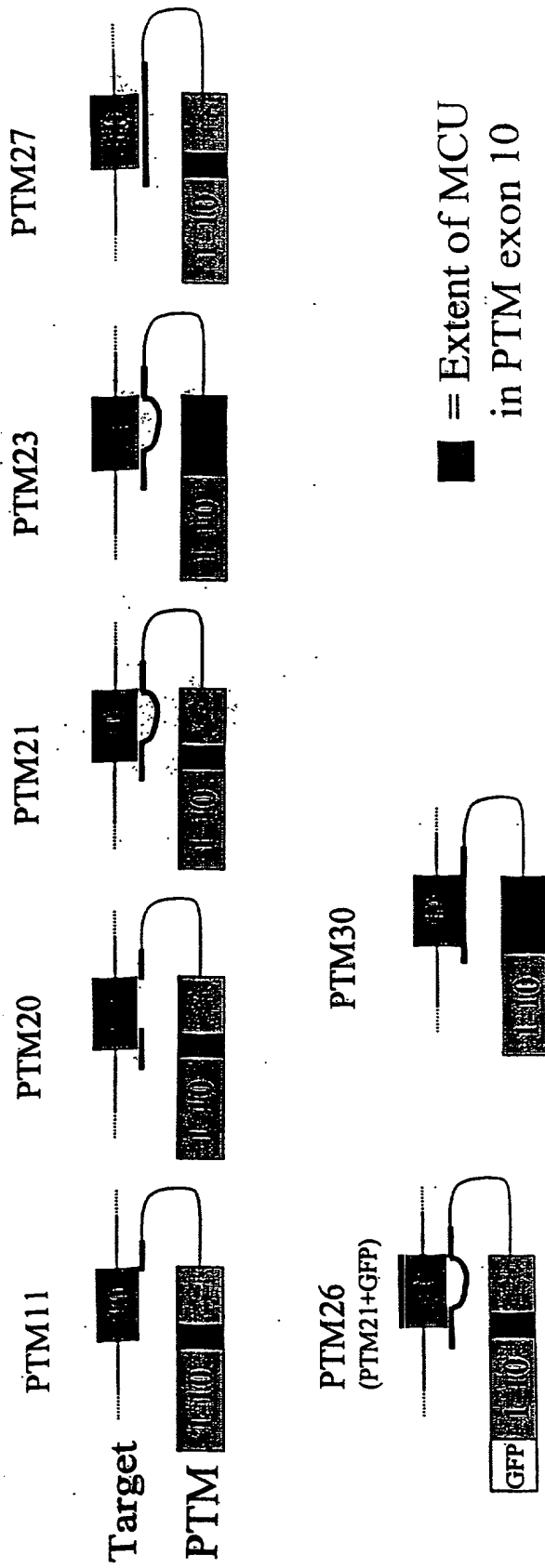


PTM with a long binding domain masking two splice sites in a mini-gene target.



PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

Figure 34



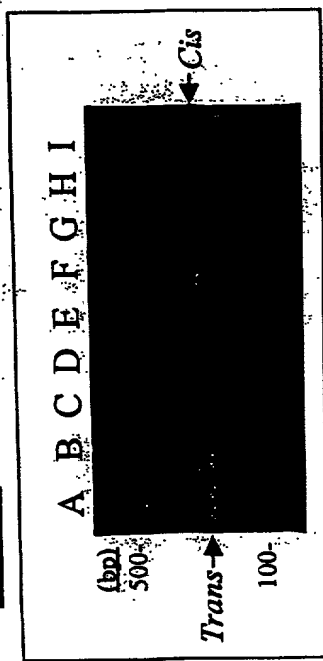
MCU in exon 10 of PTM
88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

ACGAGCTTGTCTCATGATCATGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAA CATTCCG
GCCGCATCAGCTTTTCAGGCCAATTTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAAGTACGACGAGTACCGCTATCGCTCGGTGATTAGGCCCTGTCAGTTGGAGGAG

Figure 35

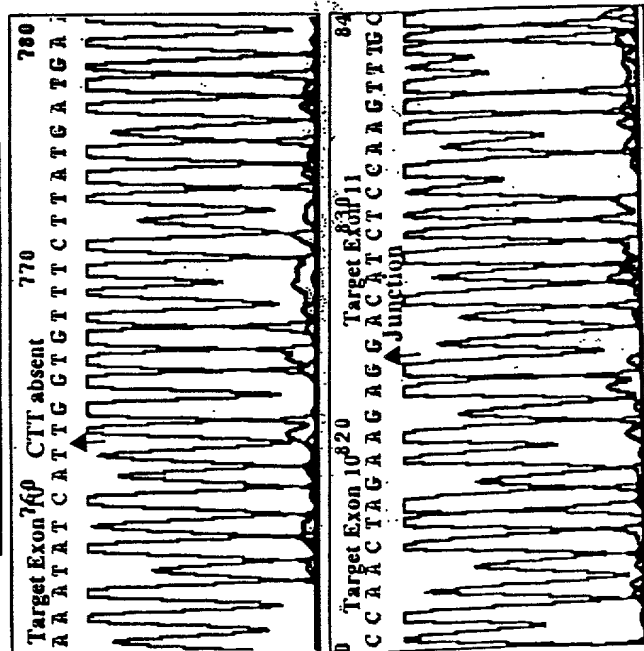
PTM

Target



46 & 89

A.
Cis-spliced product
[Primers CF1 + CF111]



B.
Trans-spliced product
[Primers CF93 + CF111]

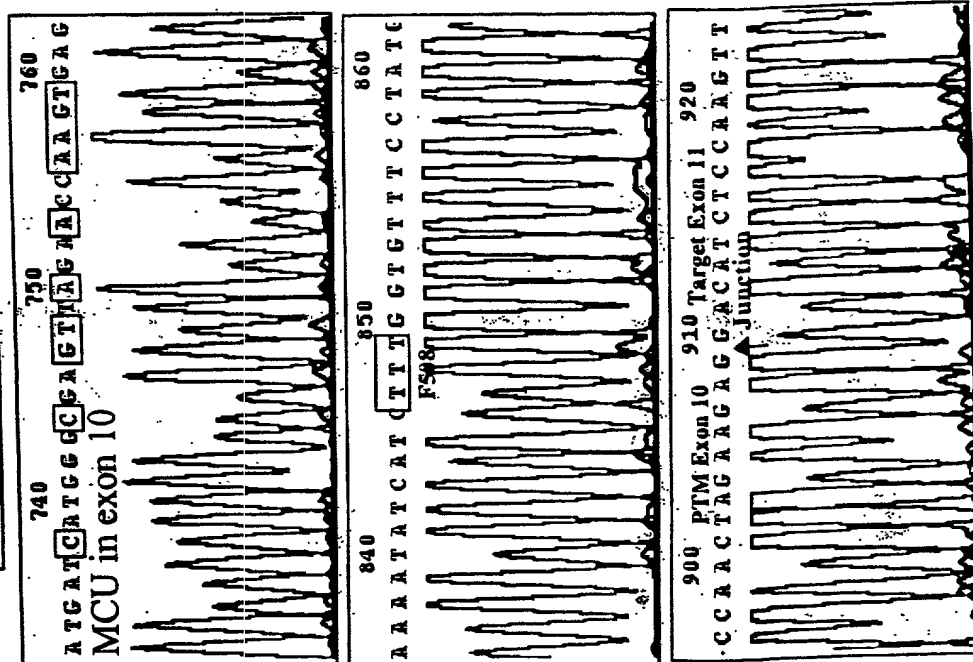


Figure 36

A

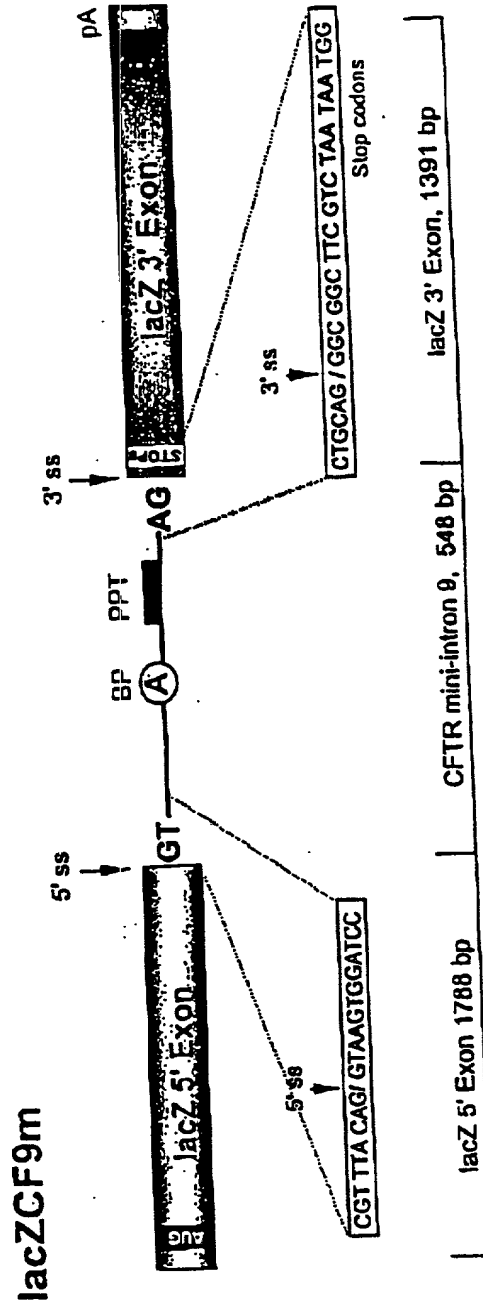
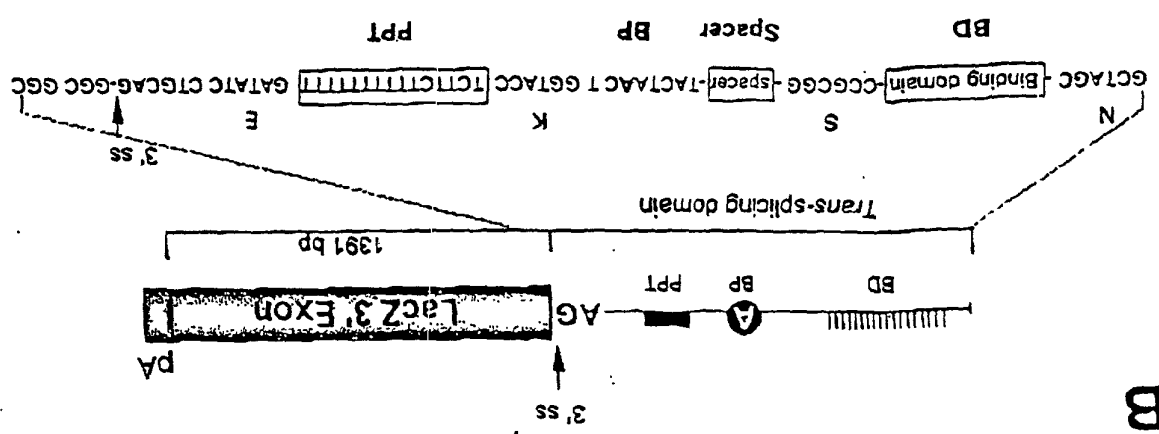
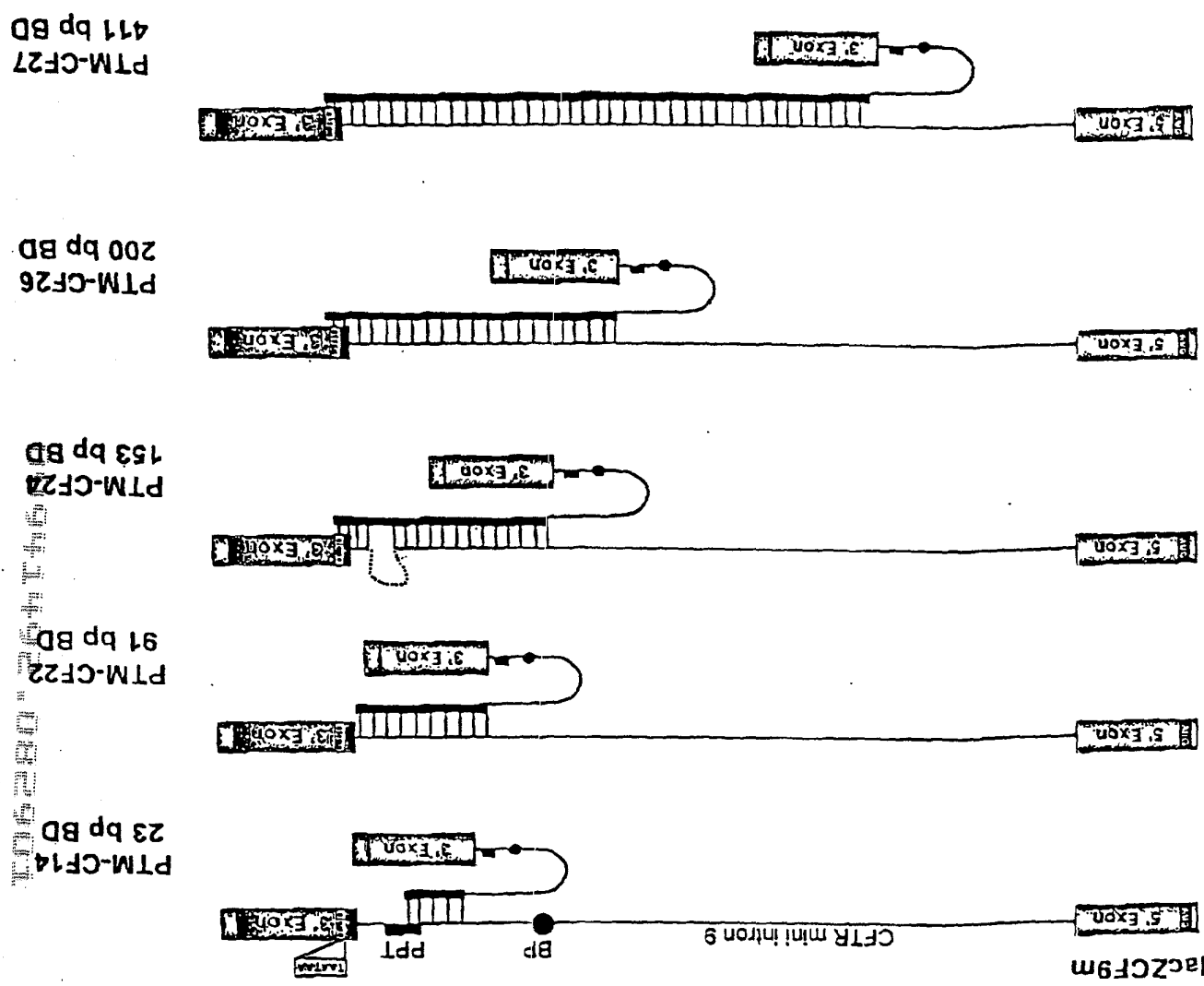


Figure 37 A

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Figure 37B



B

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C

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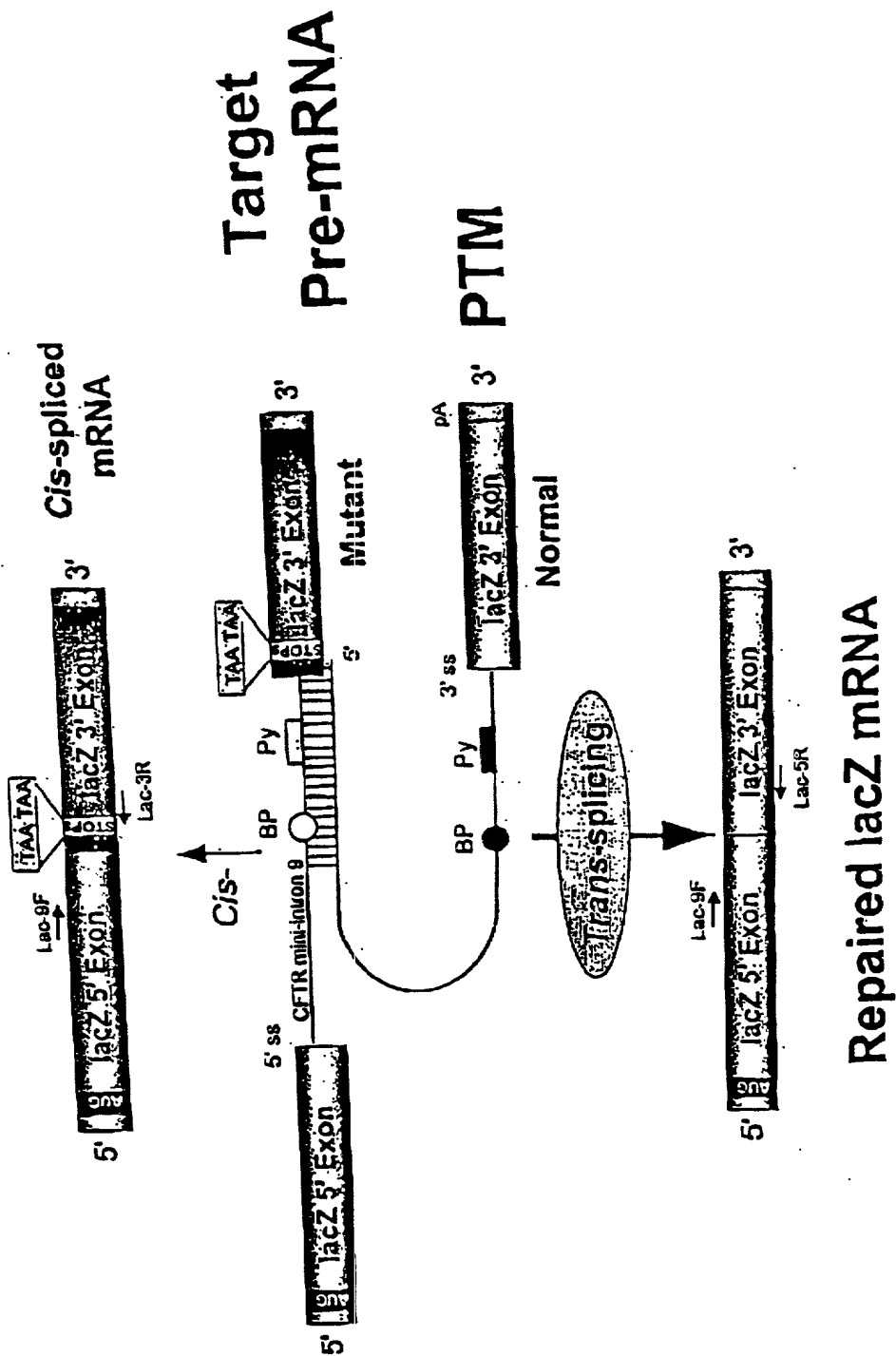


Figure 37C

106230" 254434660

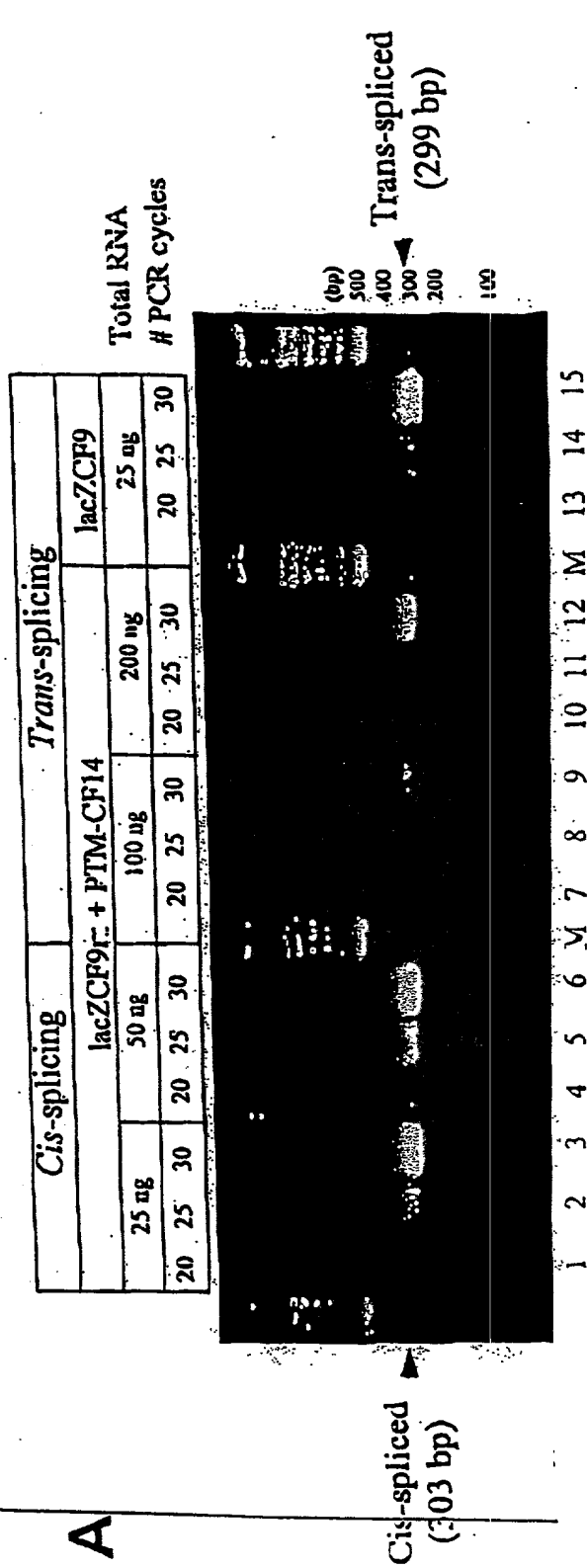
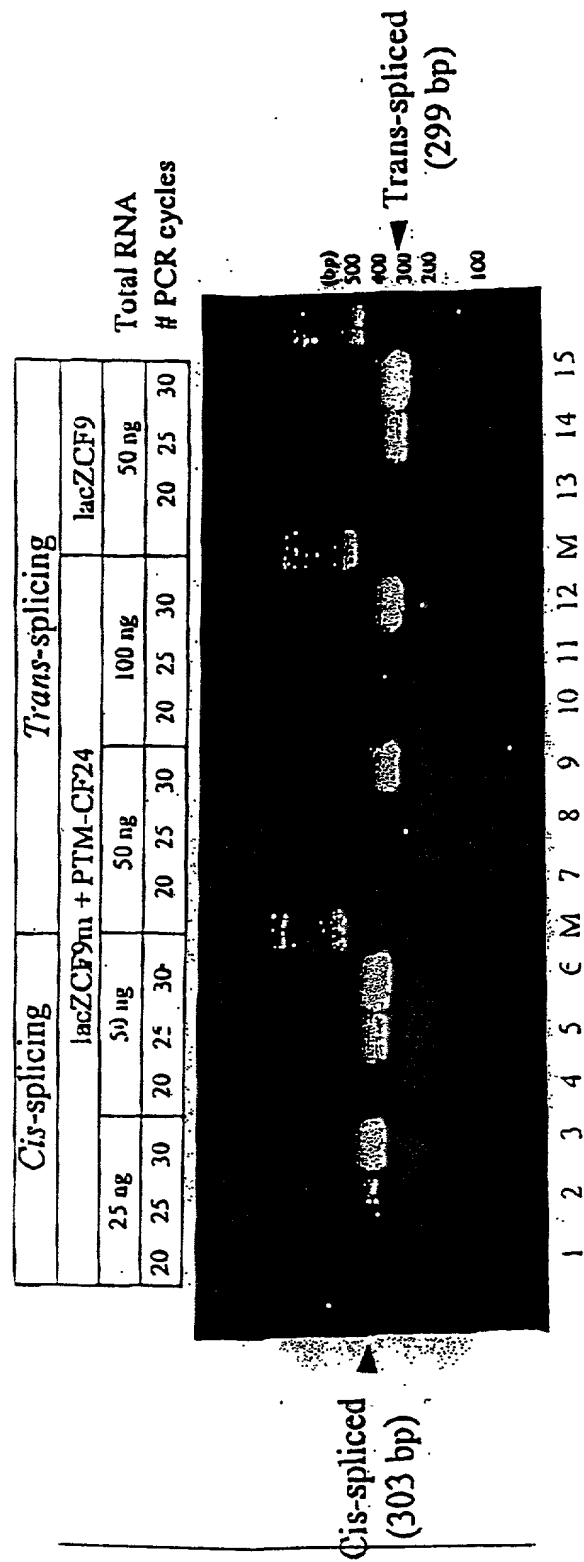


Figure 38A



B

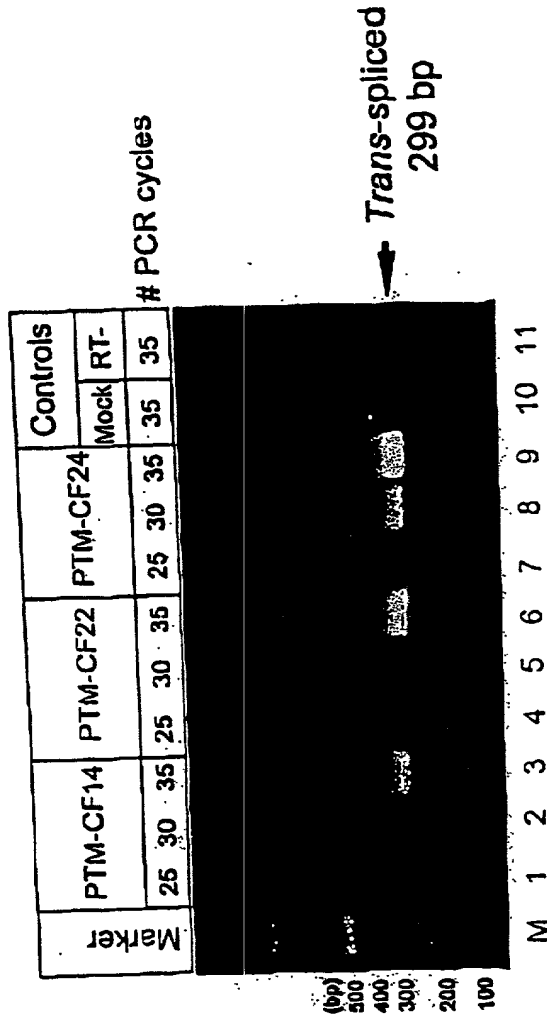


Figure 38B

106280 * 2644660

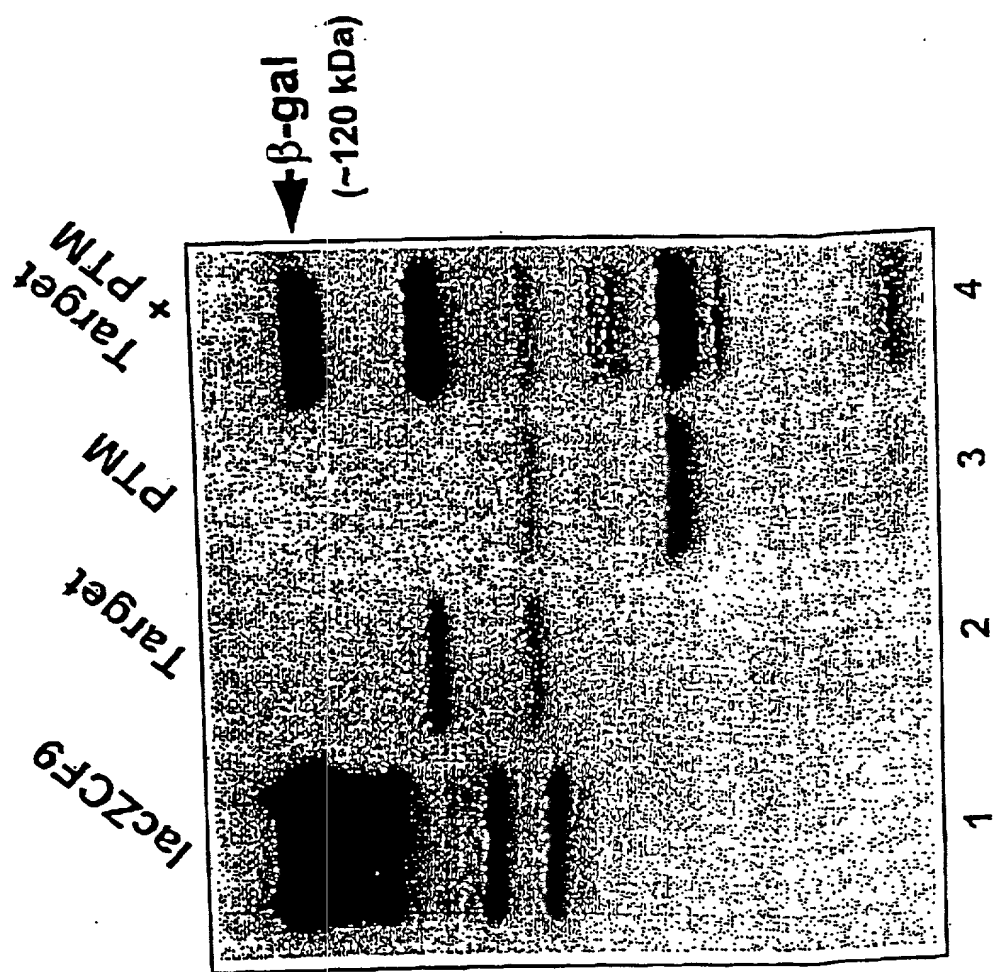
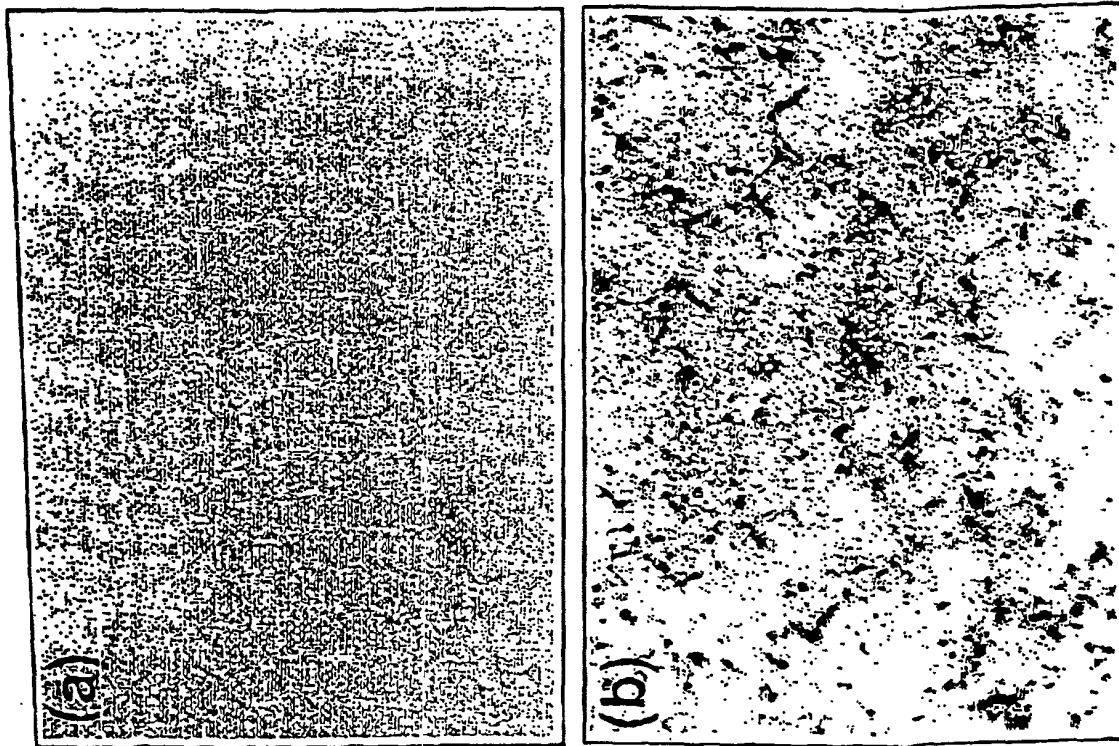


Figure 39

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Figure 40A



TOP SECRET 25414660

B

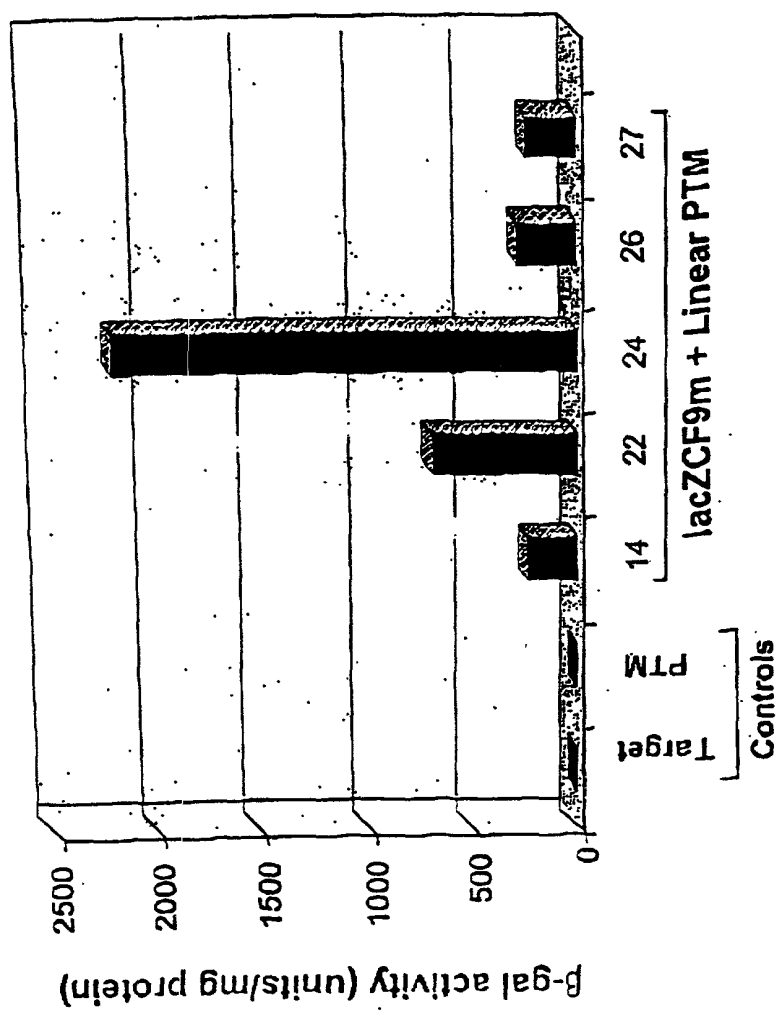


Figure 40B

C

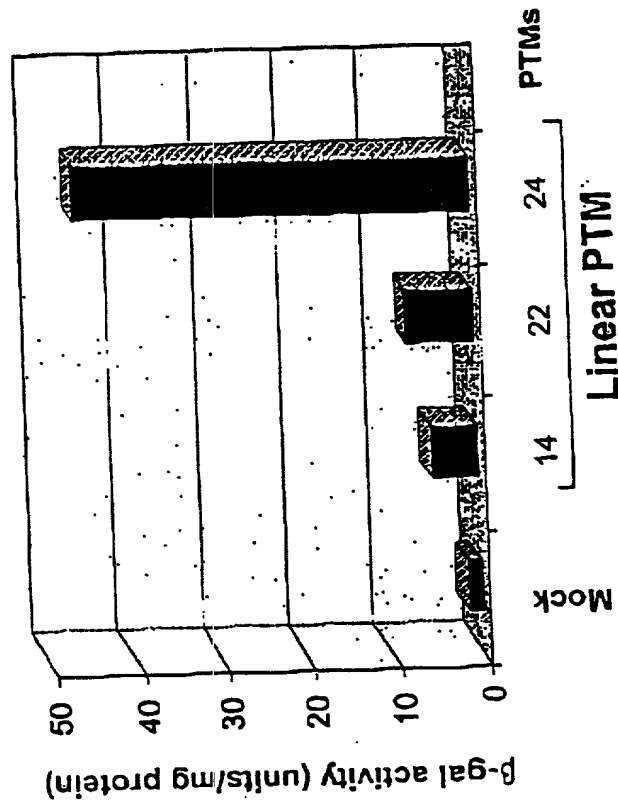


Figure 40C

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A

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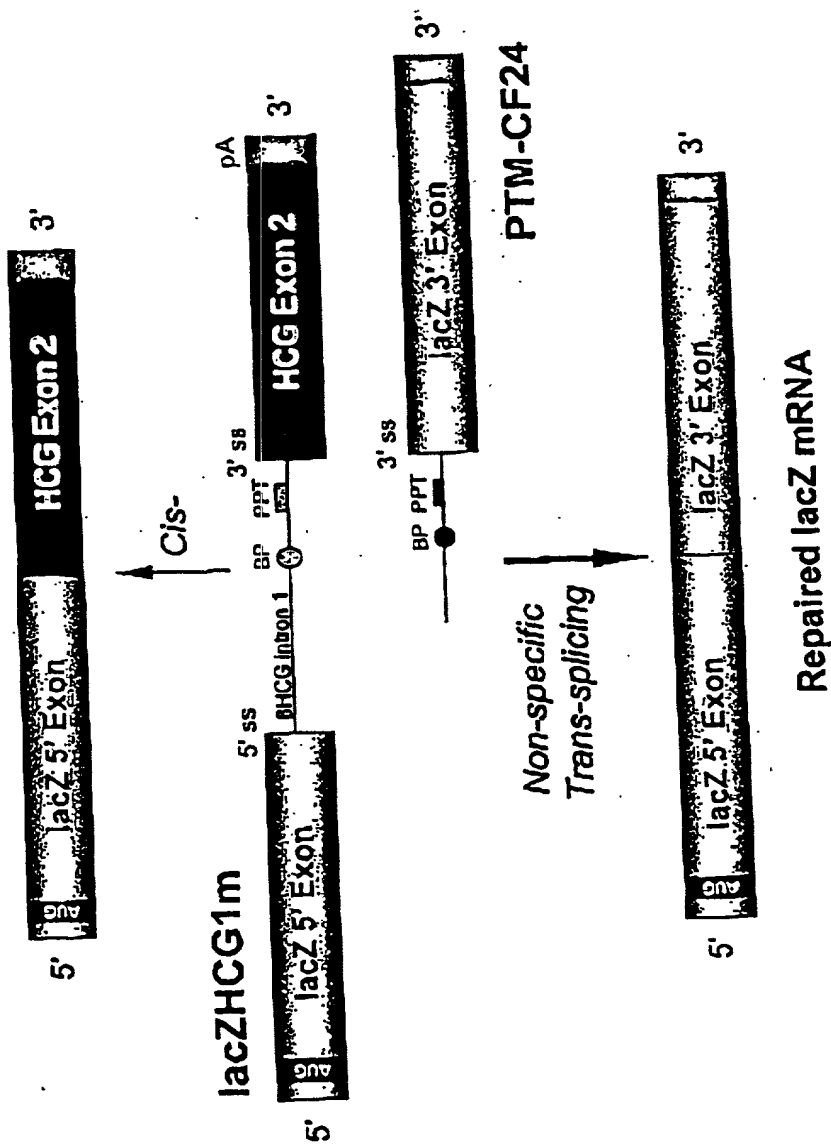


Figure 41A

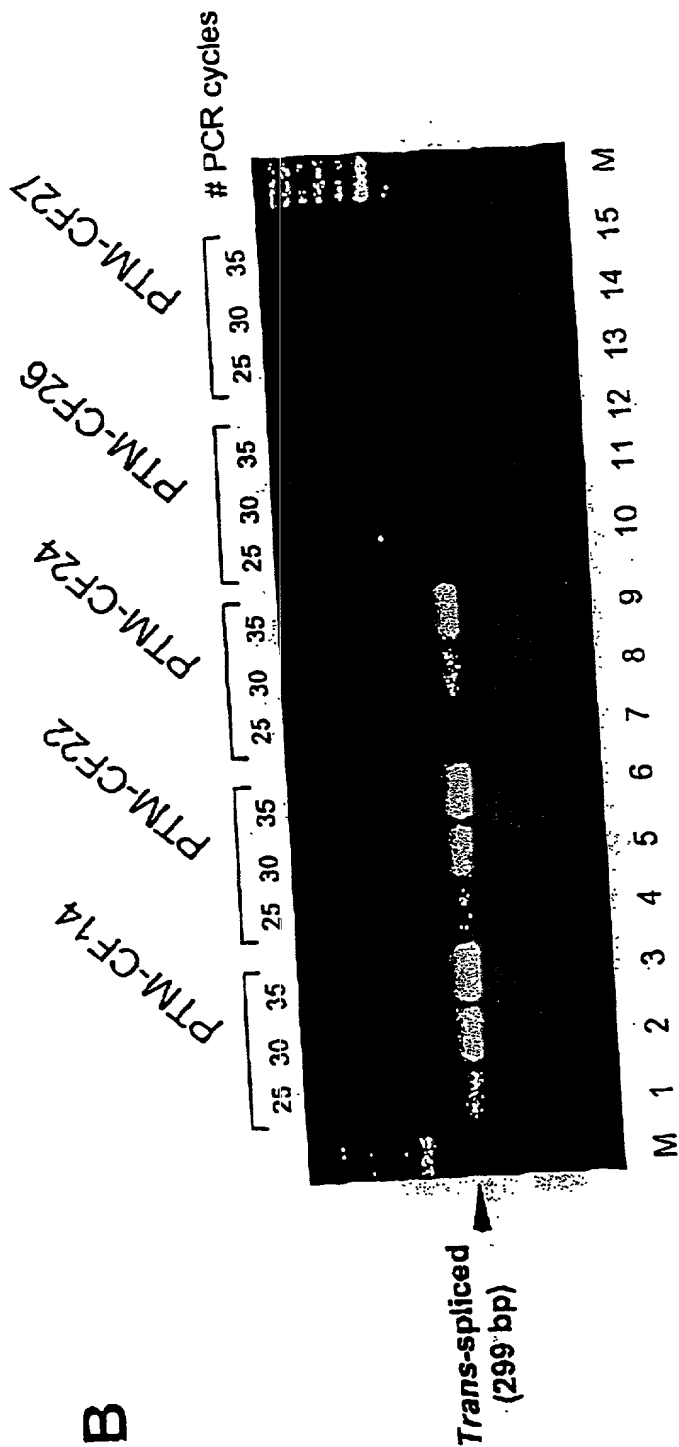


Figure 4B

C

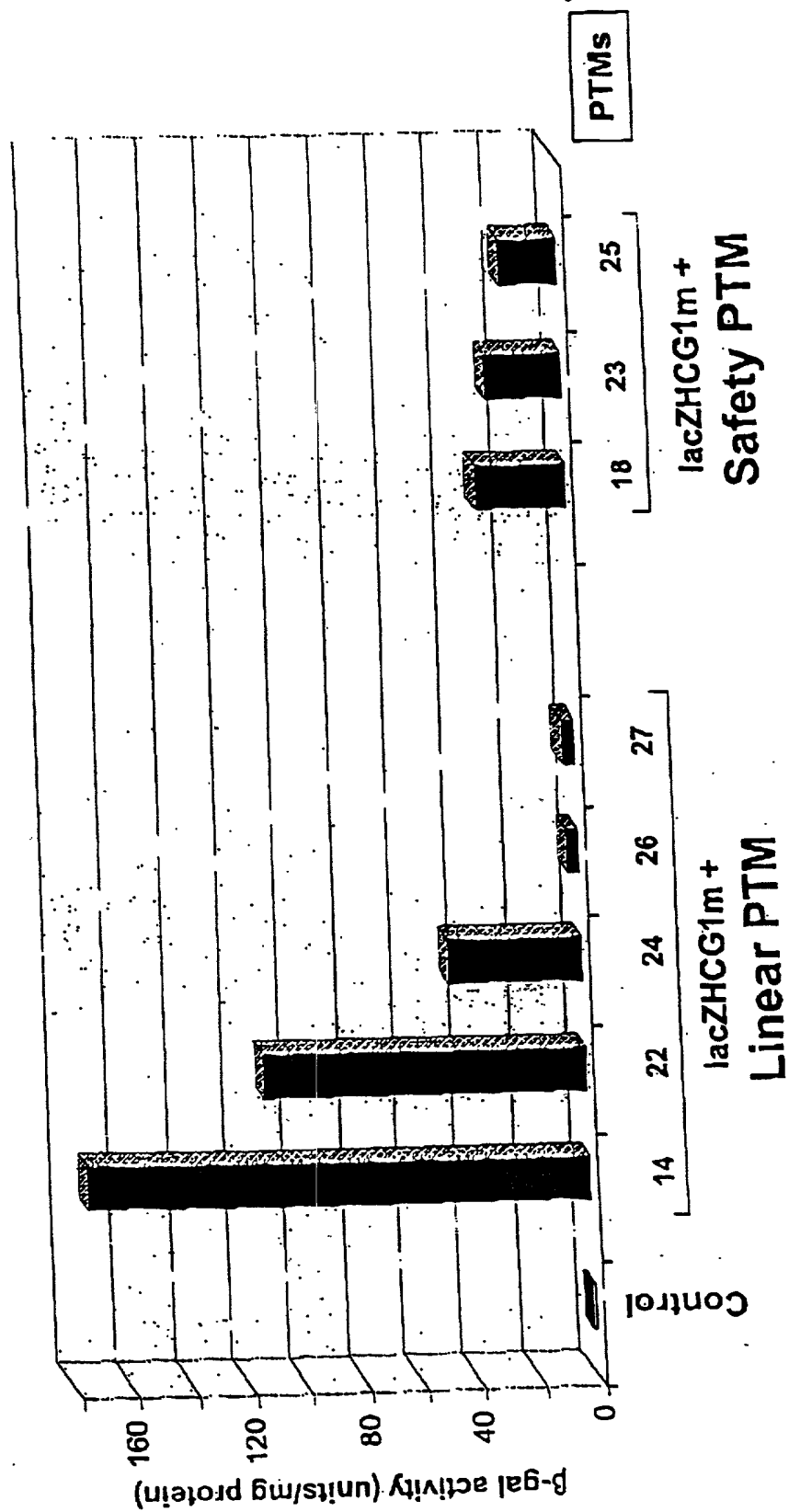


Figure 4C

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Exons 1-10

ATGCAGAGGTGCGCTCTGGAAAAGGCCAGCGTTGTCTCCAACTTTTTTTCAGCTGGACCAGACCAATTTTGAGGAAAG
GATACAGACAGCGCCTGGAATTGTGAGACATATACCAAATCCCTTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT
GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCTAACTCATTAATGCCCTTCGGCGATGTTTTTCTGG
AGATTTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTACCAGAGCAGTACAGCCTCTCTTACTGGGAAGAATCA
TAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTAT
TGTGAGGACACTGCTCCTACACCCAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGT
TTGATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACCTTGTAGTCTCCTTT
CCAACAACCTGAACAAATTTGATGAAGGACTTGCAATGGCACATTTCTGTGGATCGCTCCTTTGCAAGTGGCACTCCT
CATGGGGCTAATCTGGGAGTTGTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCTGTAGTCTCTTGGCCCTTTTTCAG
GCTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAAAATGATTGAAAACCTTAAGACA
AACAGAACTGAAACTGACTCGGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCCTCCGGAAAAATATTCACCACCATCTCATTCT
GCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAA
CAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
AATGTAACAGCCTTCTGGGAGGAGGGATTGGGGAATTATTTGAGAAAGCAAAACAAAACAATAACAATAGAAAAACTT
CTAATGGTGATGACAGCCTCTTCTTCACTAATTTCTCACTTCTTGGTACTCCTGTCTGAAAGATATTAATTTCAAGAT
AGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGCTCATGATGATCATGGGCGAG
TTAGAACCAAGTGAAGGCAAGATCAAACTTCCGGCCGCATCAGCTTTTGAGCCCAATTGAGTTGGATCATGCCCGGTA
CCATCAAGGAGAAACATAATCTTCGGCGTCAAGTACGACGAGTACCGCTATCGCTCGGTGATTAAAGCCTGTGAGTTGGA
GGAG


Trans-splicing domain

GTAAGATATCACCGATATGTGTCTAACCTGATTGGGCGCTTCGATACGCTAAGATCCACCGG
TCAAAAAGTTTTACATAATTTCTTACCTCTTCTTGAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATCATTG
GAAACACCAATGATATTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATCTTCCACTGT
GCTTAATTTTACCCTCTGAATTTCTCCATTTCTCCATAATCATCATTACAACCTGAACTCTGGAAATAAAACCCATCATT
ATTAACCTCATTATCAATCACGCT

SCANNED, #

Figure 42

153 bp PTM24 Binding Domain:

Nhe I 153 bp BD underlined
GCTAGC-GACGAAGCGCCCTCAGCTCAGGATTCACCTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATA
TTCTTATTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II
AC-CCGCGG

68 7 09

Figure 43 A

Trans-splicing domain

AATAATGACGAAGCCGCCCTCAGGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGATATTCTTA
TTTGTAAGATTCTATTAAGTCACTTGGATTCAAAATATTTAAATACTTCTGTTCACCTACTCTGCTATGCCCGC
GGAACATTATTATAACGTGCTCGAATACTAAGGTACCTCTTCTTTTTTTTGATATCCTGCAG

Exons 10-24

ACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGAAGAATTTCACTCT
 GTTCTCAGTTTTCTGGATTATGCCCTGGCACCATTAAAGAAAATATCATCTTGGTGTTCCTATGATGAATATAGATA
 CAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGAGAGAAAAGACAATATAGTTCTTGGAGAA
 GGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATT
 TATTAGACTCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAACTGATGGC
 TAACAAAAGTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAATATTAATTTGTCATGAAGGT
 AGCAGCTATTTTTATGGGACATTTTCAAGAACTCCAAAATCTACAGCCAGACTTTAGCTCAAACTCATGGGATGTGATT
 CTTTCGACCAATTTAGTGAGAAAGAAGAAATCAATCCTAAGTGAACCTTACACCGTTTCTCATTAGAAGGAGATGC
 TCCTGTCTCCTGGACAGAAACAAAAACAATCTTTAAACAGACTGGAGAGTTTGGGGAAAAAGGAAGAATTTCTATT
 CTCAATCCAATCAACTCTATACGAAAATTTTCCATTGTGCAAAAGACTCCCTTACAAATGAATGGCATCGAAGAGGATT
 CTGATGAGCCTTTAGAGAGAAGGCTGTCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACTGCCCTCGCATCAGCGT
 GATCAGCACTGGCCCCAGCTTCAGGCACGAAGGAGGAGTCTGTCTGAACCTGATGACACACTCAGTTAACCAAGGT
 CAGAACATTCACCGAAAGACAACAGCATCCACAGAAAAGTGTCACTGGCCCCCTCAGGCAAACTTGACTGAACTGGATA
 TATATTCAAGAAGGTTATCTCAAGAACTGGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTT
 TTTTGATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTTA
 ATTTTTGTGCTAAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCTTTCTTGGTTGTGCTGTGGCTCCTTGGAA
 ACACCTCTCTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTC
 GTATTATGTGTTTTACATTTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCACTGGTG
 CATACTCTAATCAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTTCTTCAAGCACCTATGTCAACCTCA
 ACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCCTTACCAT
 ATTTGACTTCATCCAGTTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTGCGAGTTTACAAACCTACATCTTTGTT
 GCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCTTCCAAACCTCACAGCAACTCAAACTGG
 AATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTTACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACG
 GCAGCCTTACTTTGAAACTCTGTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTAACCTGTCAACACTG
 CGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCACTCTTCTTCACTGCTGTTACCTTCATTTCCATTTTAAACAG
 GAGAAGGAGAAGGAAGAGTTGGTATTATCTGACTTTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAACTC
 CAGCATAGATGTGGATAGCTTGATGCGATCTGTGAGCCGAGTCTTTAAGTTTCACTGACATGCCAACAGAAGGTAAACCT
 ACCAAGTCAACCAACCATACAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATG
 ACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACAGCAAAATACACAGAAGGTGGAATGCCATATTAGA
 GAACATTTCTTCTCAATAAGTCTTGGCCAGAGGTGGGCTCTTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTA
 TCAGCTTTTTTGGAGACTACTGAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAAC
 AGTGGAGGAAGCCTTTGGAGTGATACACAGAAAGTATTTATTTTTCTGGAACATTTAGAAAACTTGGATCCCTA
 TGAACAGTGGAGTGATCAAGAAATATGGAAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTTTCTTGGG
 AAGCTTGACTTTGTCTTGTGGATGGGGCTGTGTCCTAAGCCATGGCCACAAGCAGTTGATGTGCTTGGCTAGATCTG
 TTCTCAGTAAGGCGAAGATCTTGCTGCTTGATGAACCCAGTGCTCATTTGGATCCAGTAACATACCAATAATTAGAAG
 AACTCTAAAACAAGCATTTGCTGATTGCACAGTAATTTCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAA
 TTTTGGTCTAGAGAAGAGAACAAGTGCGGCAGTACGATTCCATCCAGAACTGCTGAACGAGAGGAGCCTCTTCCGGC
 AAGCCATCAGCCCTCCGACAGGGTGAAGCTCTTCCCCACCGGAATCAAGCAAGTGCAAGTCTAAGCCCCAGATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTCATCATCATCATCATCAT

SCANNED, #

Figure 43B

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106280"2644660

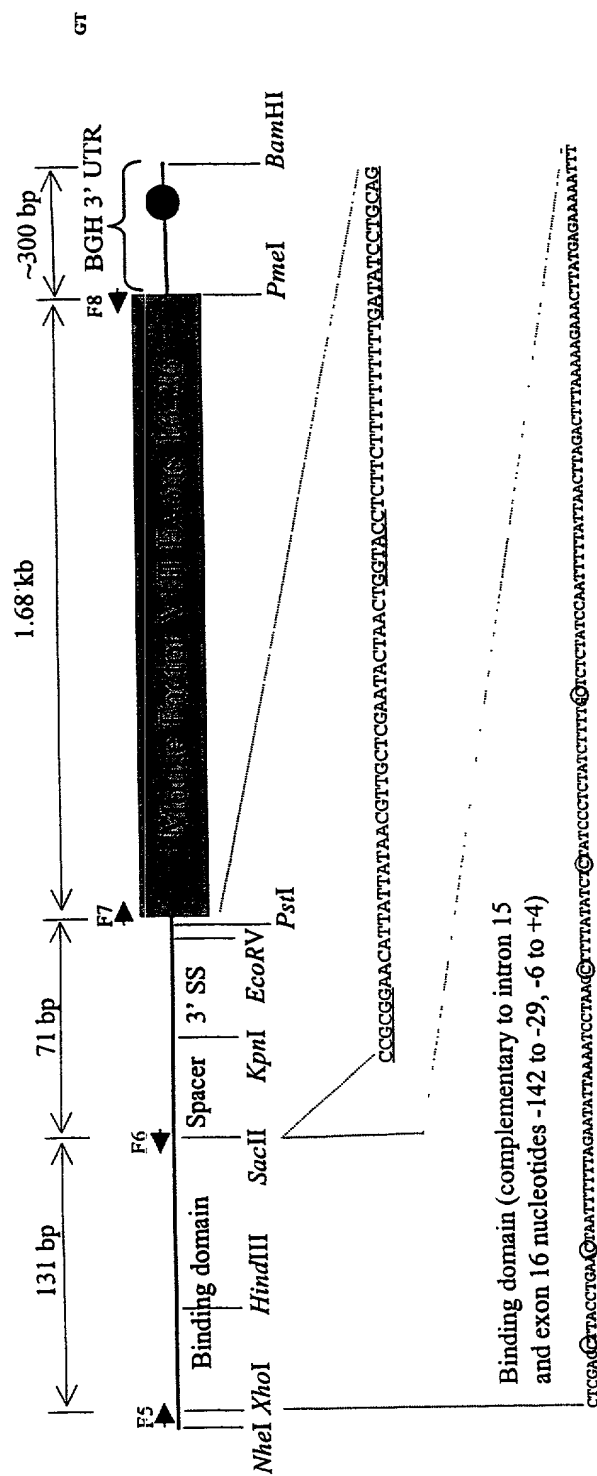


Figure 44 A

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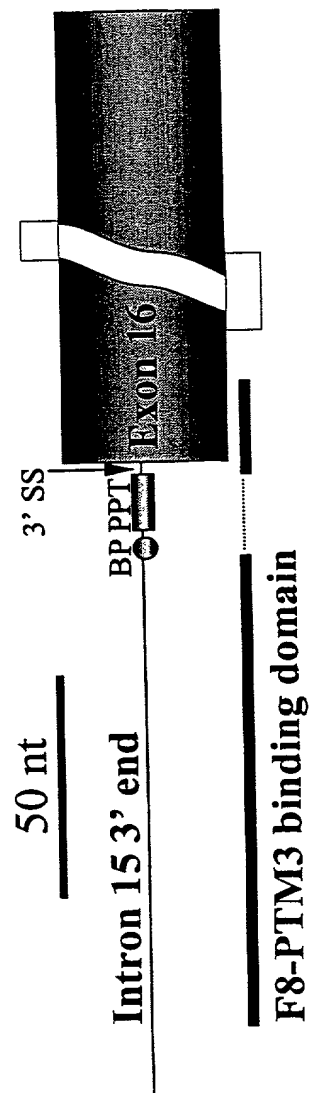
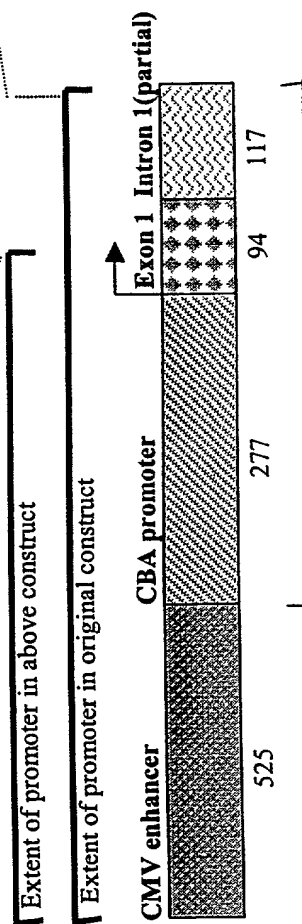


Figure 44 B

Nucleotide changes are shown in blue
 Boxed = CAT box, TATA box
 Boxed + Arrow = Transcription Start
 Oval = Downstream elements
 Bold = Binding domain
 Italicized = Spacer+ppT+BP+AG dinucleotide

Sequence not included in construct

CGCCGCCCTCGGCGCGCCGCCCGGCCTTGACTGACCGCGTTACTCCACAGGTGAG
CGGGCGGGACGGCCCCCTTCTCCTCCGGGCTGTAAATTAGCGCTTGTTTTTAATGA CCGCT

$$\begin{aligned} \mathbf{F13} + \mathbf{F2} &= 235 + 106 = 341 \text{ bp} \\ \mathbf{F13} + \mathbf{F4} &= 235 + 315 = 550 \text{ bp} \end{aligned}$$


Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

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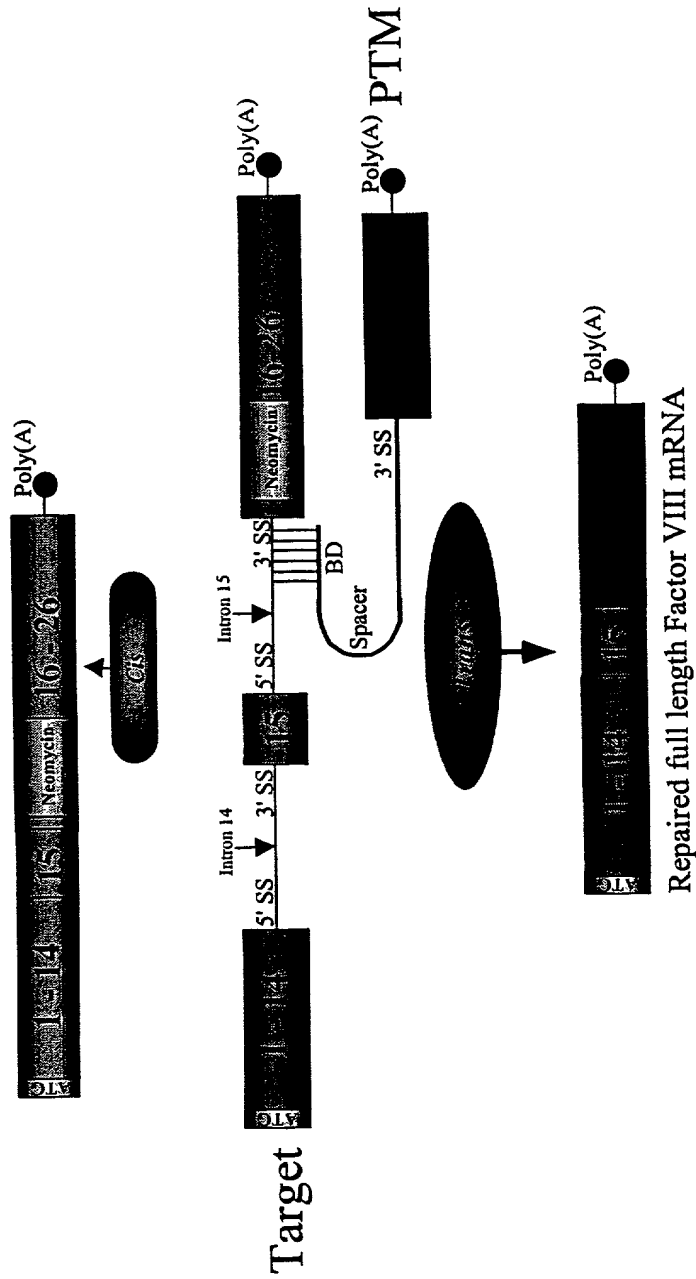
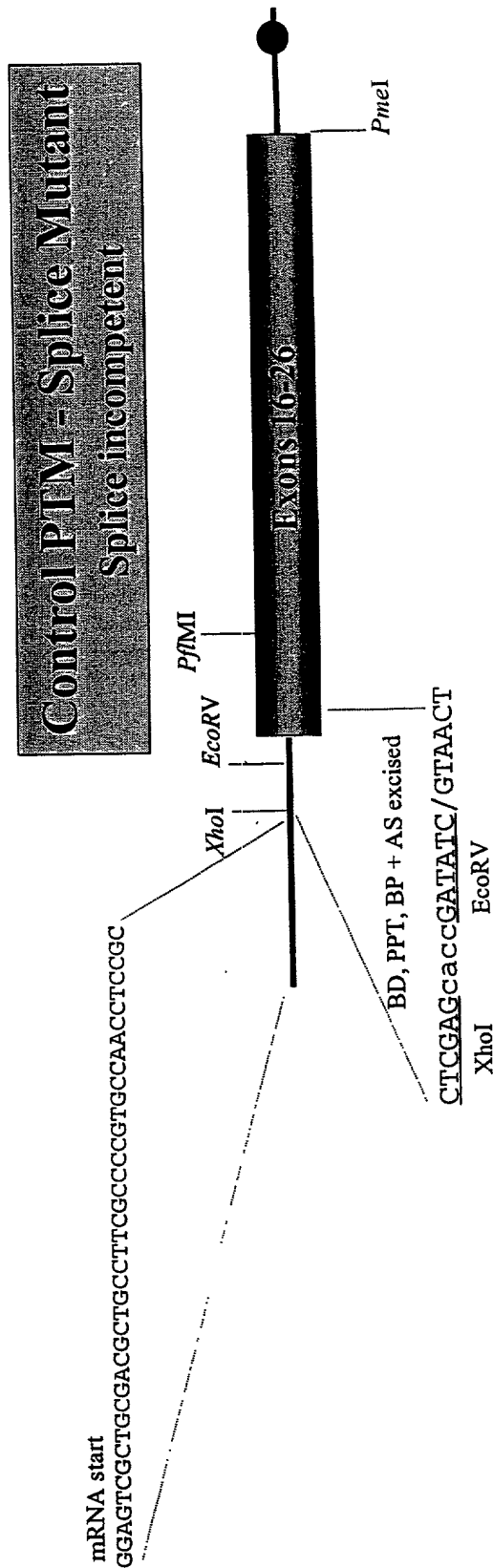


Figure 44D

Figure 45



Method:

Excise TSD and part of exon 16 with

XhoI and PflMI and ligate in a PCR product that:

1) eliminates the TSD and splice acceptor site

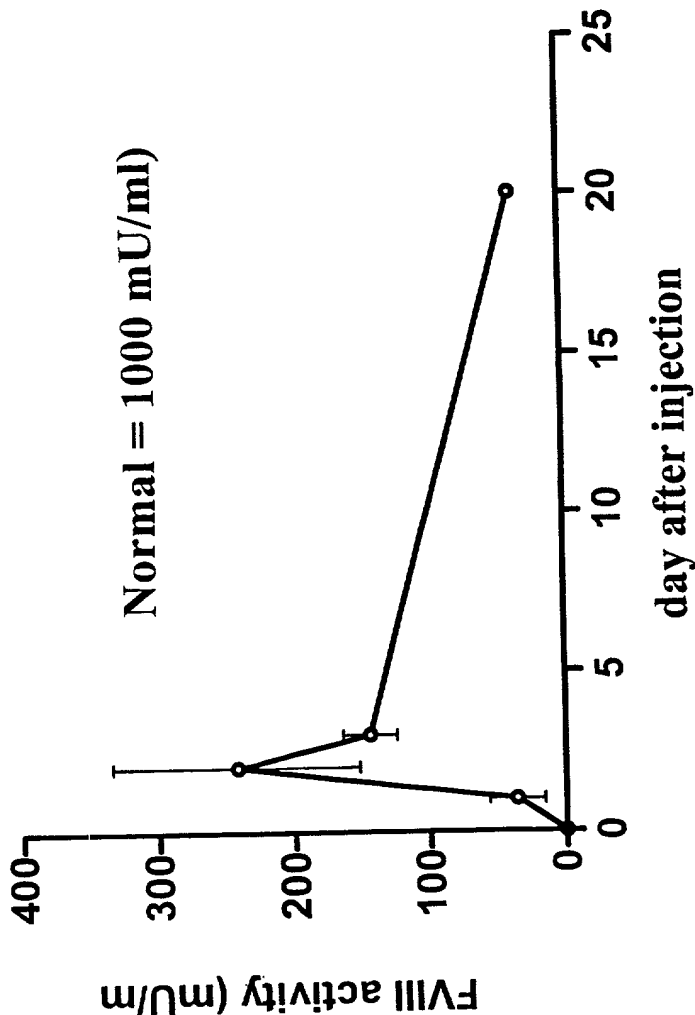
2) inserts EcoRV adjacent to exon 16

3) restores the coding for exon 16

Repair of Factor VIII

Preliminary results from one experiment

FVIII activity in Exon 16 FVIII-KO mice
after IV PTM-FVIII intraportal infusion
(100ugDNA)(n=3)



METHODS

Inject plasmid intraportally

↓

Sample blood (1, 2, 3, 20 d)

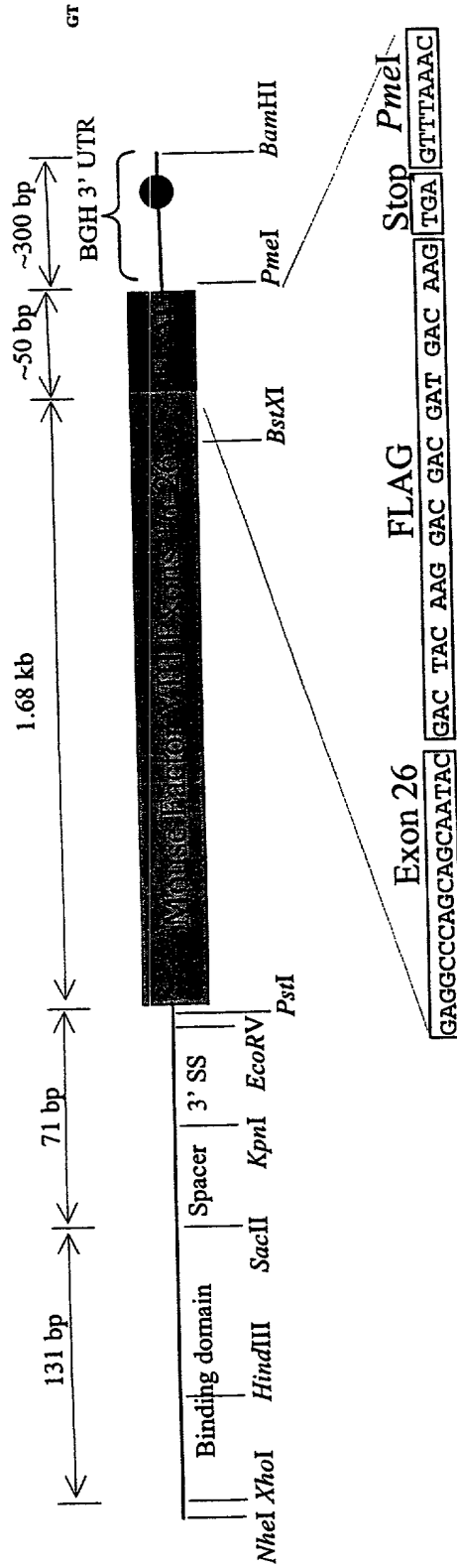
↓

Assay for factor VIII activity

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Figure 46

Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26 and a C-terminal FLAG tag. BGH = bovine growth hormone 3' UTR; Binding domain = 125 bp.

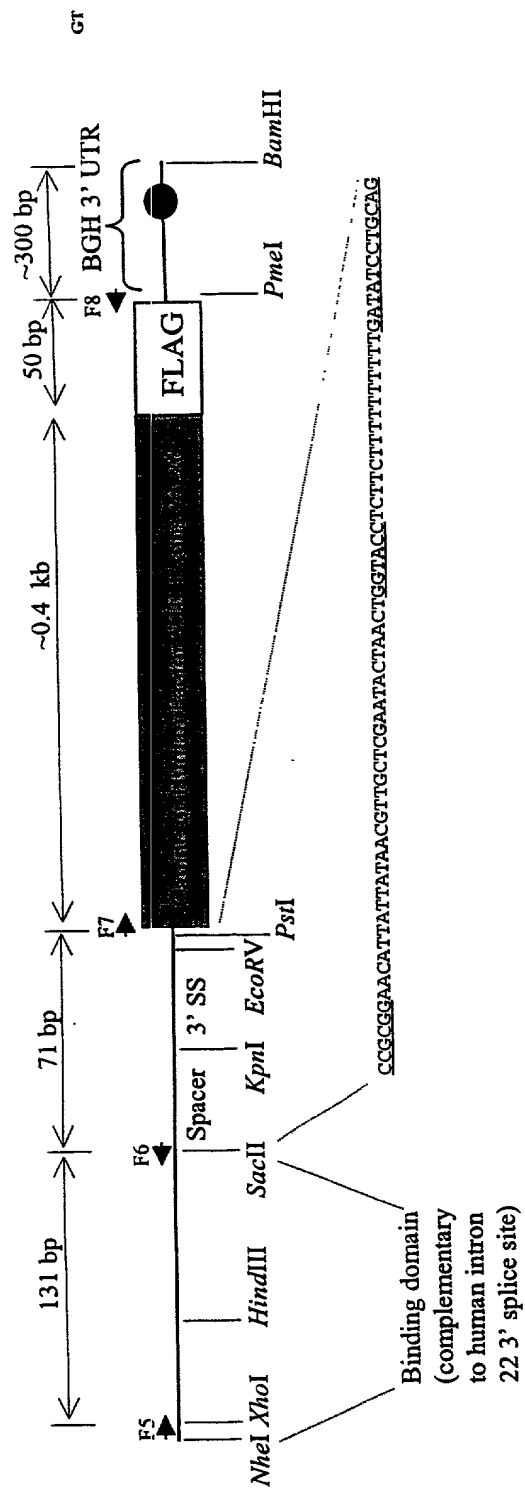


REFERENCE FOR DESIGN OF FLAG TAG

Brann T, Kayda D, Lyons RM, Shirley P, Roy S, Kaleko M, Smith T.
Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates.
Hum Gene Ther 1999 Dec 10;10(18):2999-3011
Genetic Therapy, Inc., a Novartis Company, Gaithersburg, MD 20878, USA.
Epitope-tagged B domain-deleted human factor VIII cDNA (flagged FVIII) was evaluated in nonhuman primates.

Figure 47A

68 76 89



FLAG = C-terminal tag to be used to detect repaired factor VIII protein.

Figure 47B

Transcription Map of HPV-16

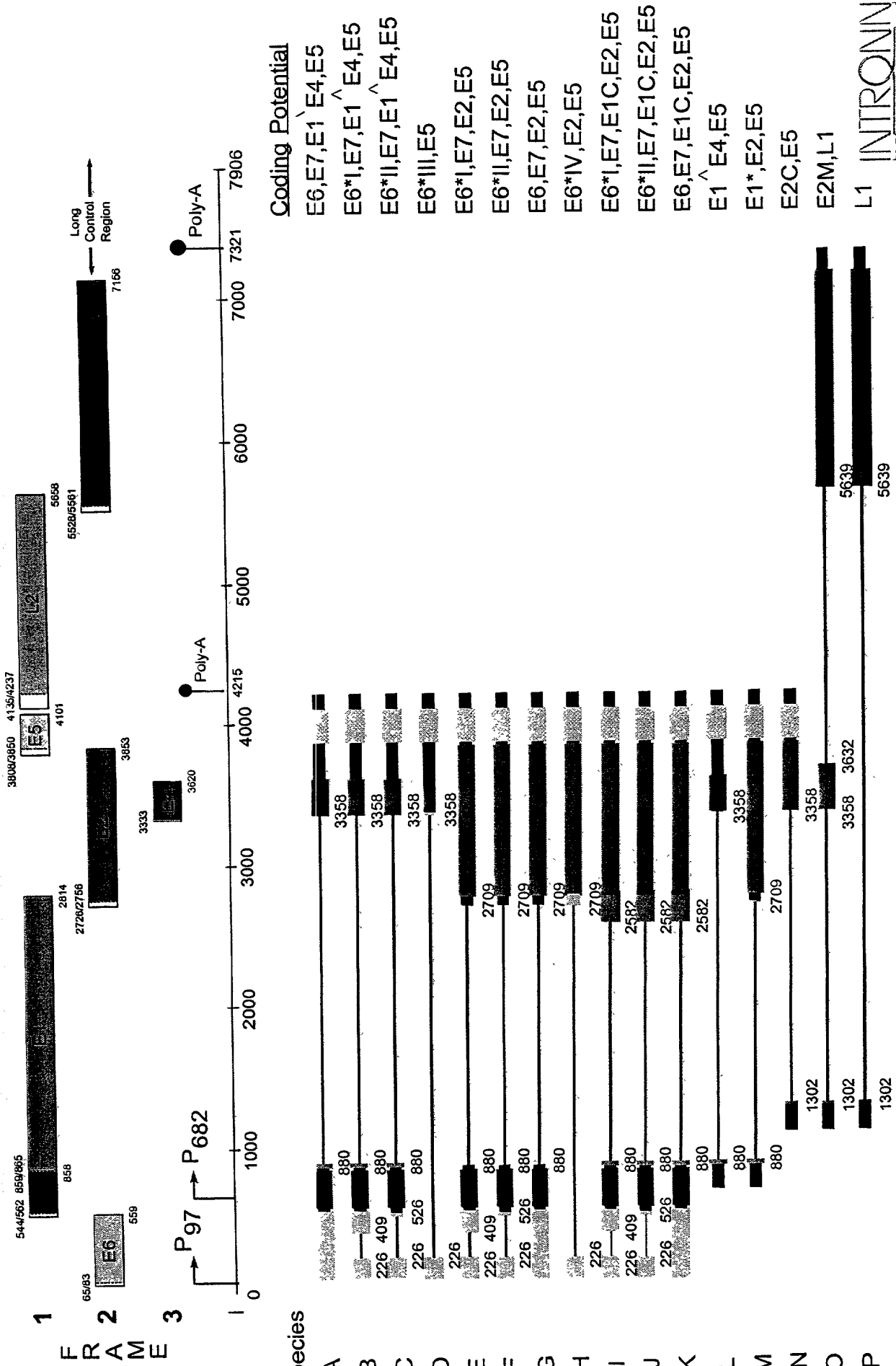
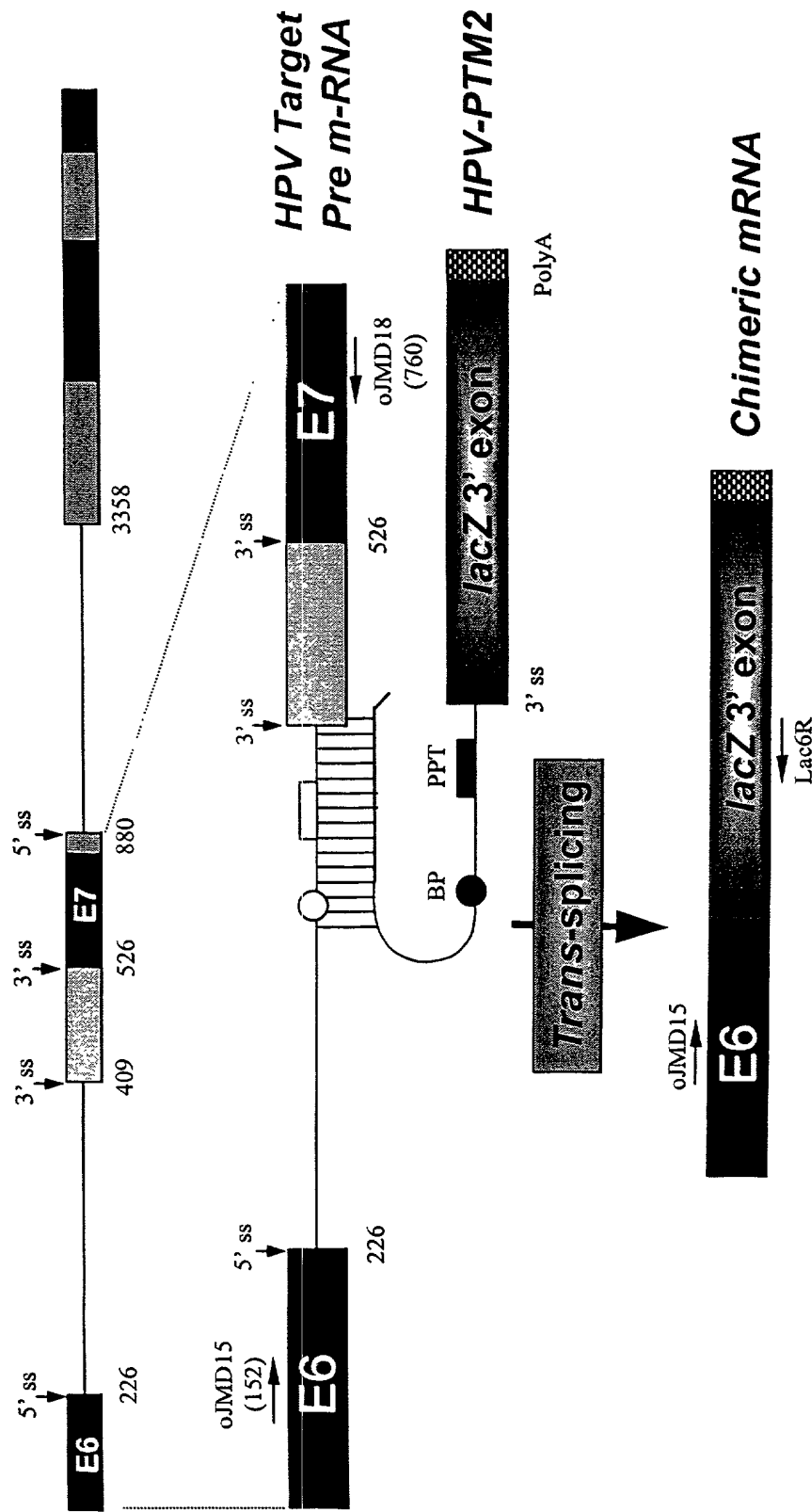


FIGURE 48

SMaRT Strategy to Disrupt the Expression of Human Papillomavirus Type 16

Targeting E6 Exon



SMaRT Strategy by 3' Exon Replacement: Schematic diagram of HPV-PTM2 binding to the 3' splice site of the HPV type 16 target pre-mRNA

FIGURE 40

NEUTRON

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PTM Design

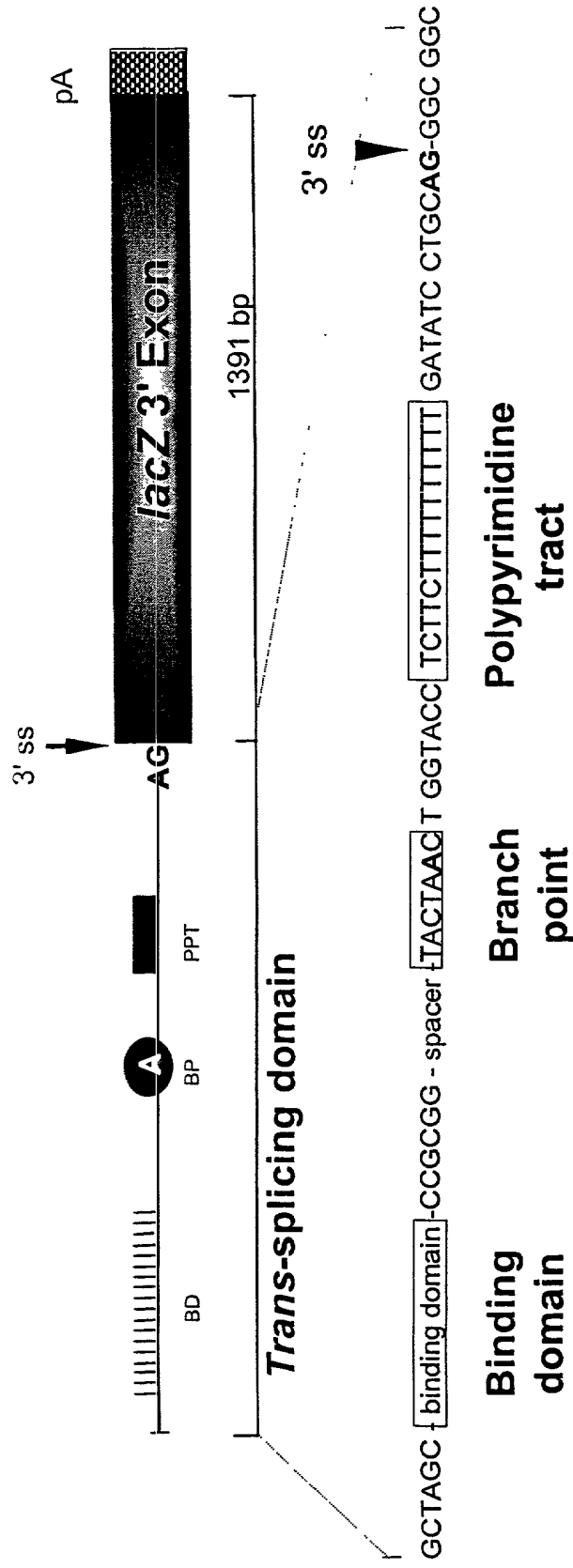
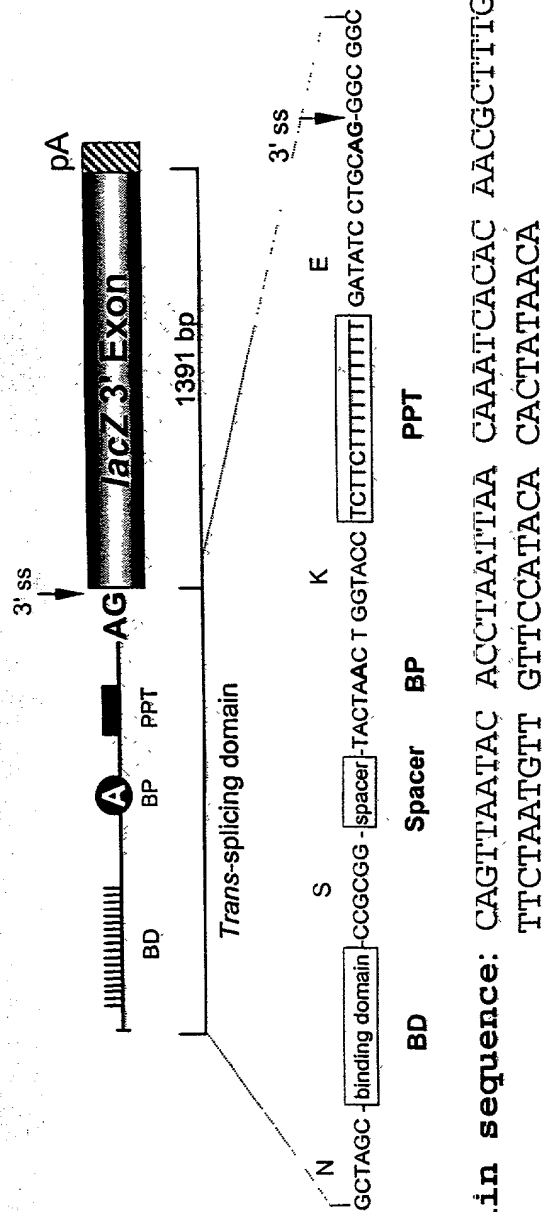


FIGURE 51

INTRONN

73 789

HPV-PTM1 with 80 bp binding domain targeted to 3' ss at 409:



A

HPV-PTM2 with 149 bp binding domain targeted to 3' ss at 409:



Binding domain sequence: CAGTTAATAC ACCTAATTA CAAATCACAC AACGCTTTGT TGTATTGCTG
TTCTAATGTT GTTCCATACA CACTATAACA ATAATGCTA TACTCACTAA
TTTTAGAATA AAACCTTTAAA CATTATATC ATACAGCATA TCGATTCCC

B

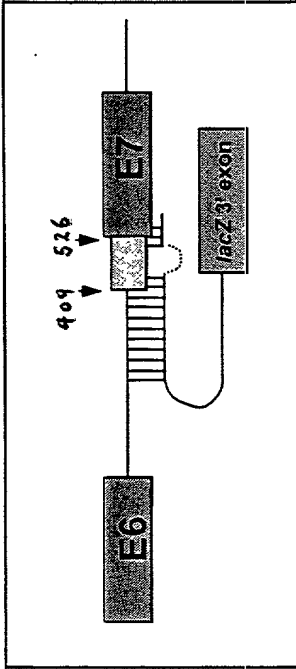
FIGURE 52

INTRONIN

Binding Domains of HPV-PTM3 and 4

HPV-PTM3 Binding domain (covers both 3' ss at 409 and 526; has 53 bp bubble)

GATGATCTGCAACAAGACATACATCGACCGGTCCA (53 nt bubble) CTTCAGGACACAGTGGCTTTTGAC
AGTTAAATACACCTAAATTAACAAATCACACACGGTTTGTGATTCGAGTTCTAATGTTGTTCCATACACACTA
TAACAAT



HPV-PTM4 Binding domain (covers both 3' ss at 409 and 526; has 76 bp bubble)

GATGATCTGCAACAAGAC (76 nt bubble) GACACAGTGGCTTTTGACAGTTAATACACCCTAATTAACAAATC
ACACAACGGTTTGTGATTCGAGTTCTAATGTTGTTCCATACACACTATAACAAT

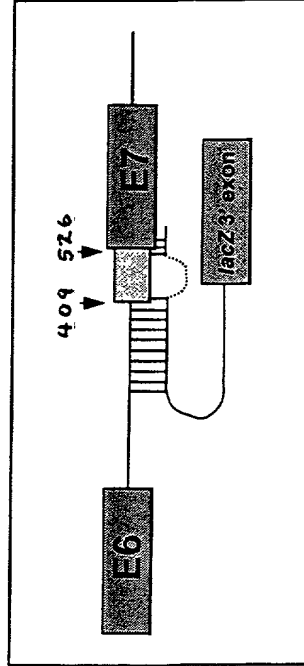


FIGURE 53

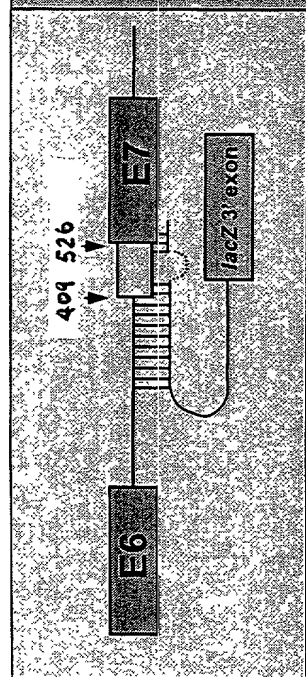
HPV-PTM5 and 6

HPV-PTM5, Binding domain (140 nt, has 53 nt bubble, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGACATACATCGACCGGTCCA. CTTGAGGACACAGTGGCTTTTGACAGTTAATACACCTAATTAACAAATCACACAACGGT
TTGTTGTATTGCAGTTCTAATGTTGTTCCATACACACTATAACA

CCGT

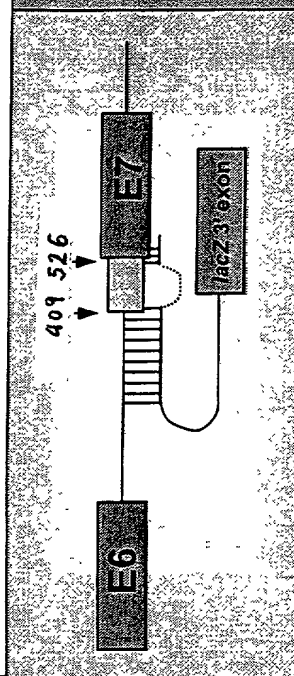
CCGT



HPV-PTM6, Binding domain (117 nt, has 76 nt bubble, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGAC.GACACAGTGGCTTTTGACAGTTAATACACCTAATTAACAAATCACACAACGGT**TTGTTGTATTGCAGTTCT**
AATGTTGTTCCATACACACTATAACA

CCGT

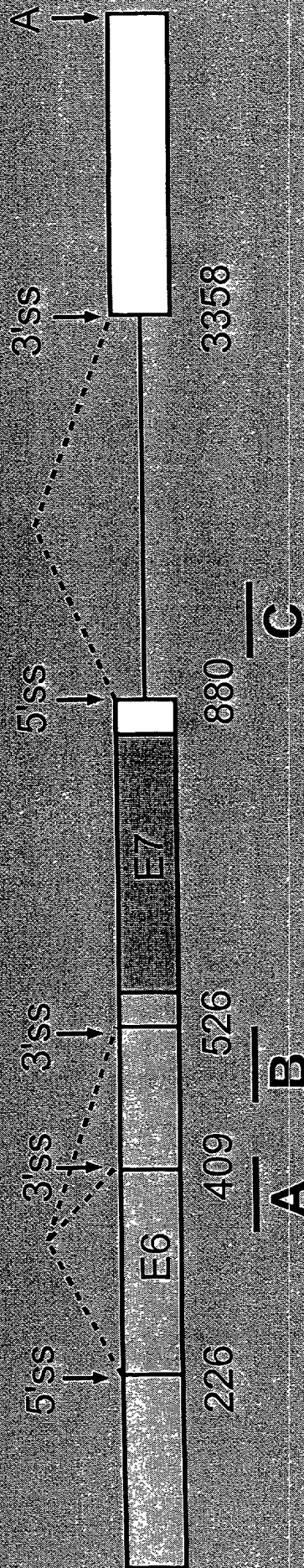


Note: Nucleotides in bold are modified to prevent PTMs cryptic splicing

FIGURE 54

INTRONN

Positions of HPV-PTM Targeting Domains



PTM	Binding Domain	
	Region	Size (nt)
HPV-PTM1	A	80
HPV-PTM2	A	149
HPV-PTM5	A+B	140
HPV-PTM6	A+B	117
HPV-PTM8	C	104
HPV-PTM9	C	174

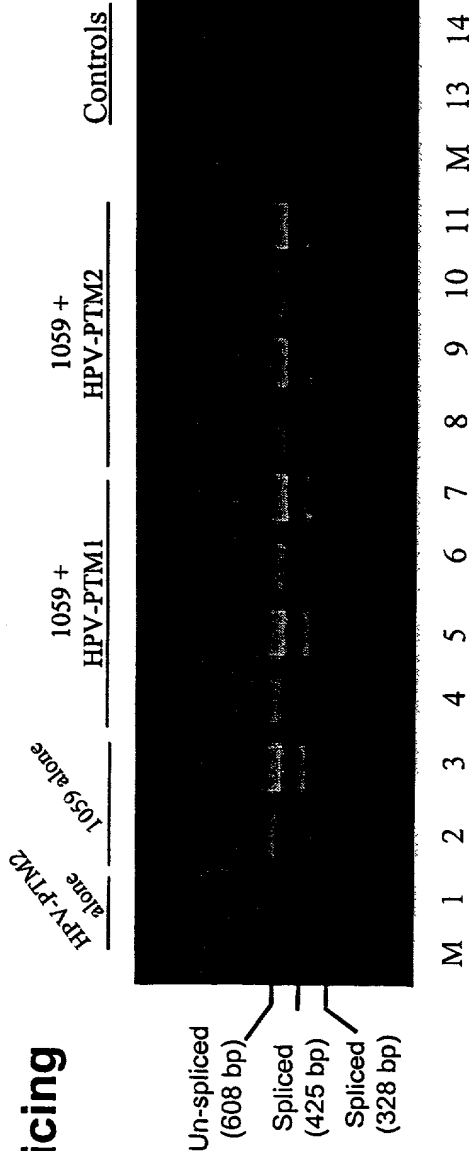
INTRONN

FIGURE 55

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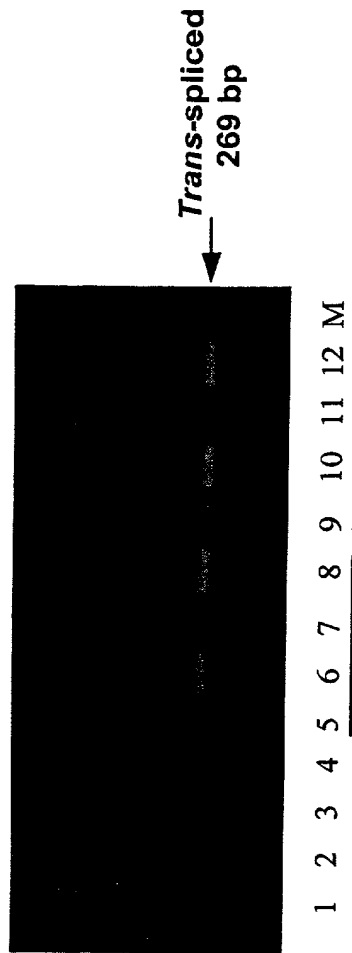
Trans-splicing Efficiency of HPV-PTMs in 293T Cells

Cis-splicing



Trans-splicing

- Mock
 - HPV-PTM1 alone
 - HPV-PTM2 alone
 - 1059 target alone
- 5 - 8: 1059 + HPV-PTM1 | 25 & 30 cycles
9 - 12: 1059 + HPV-PTM2



RT-PCR Analysis of total RNA

INTRONN

FIGURE 56

Trans-splicing between target pre-mRNA and PTM is accurate (293T cells)

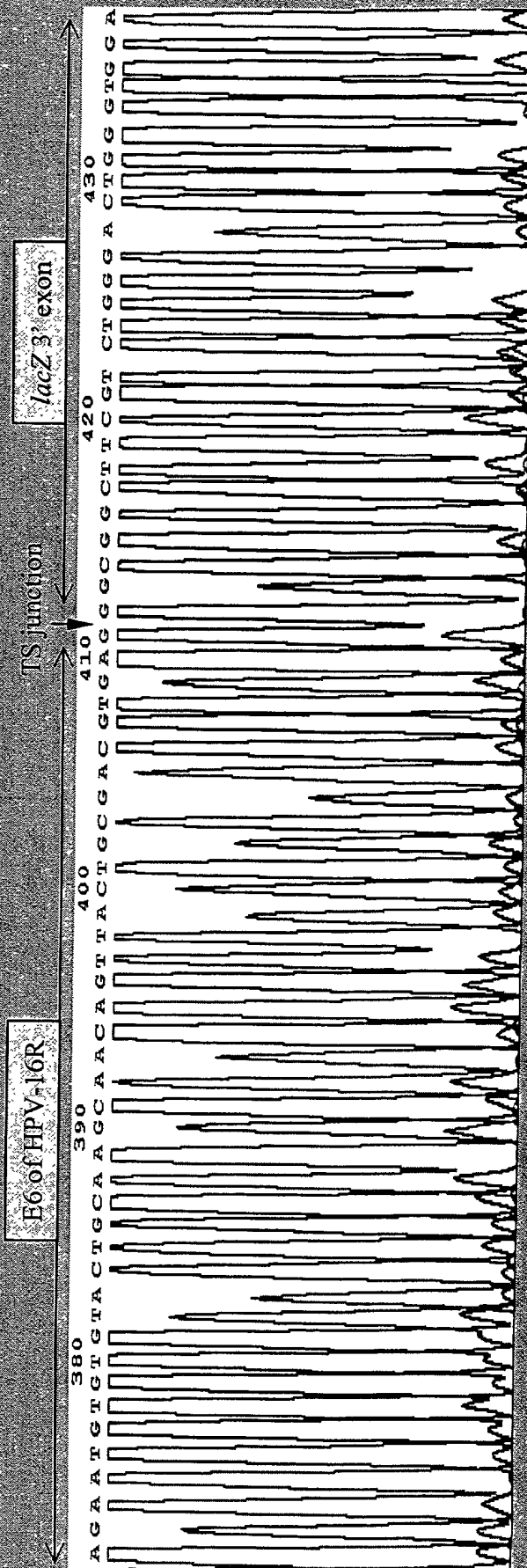
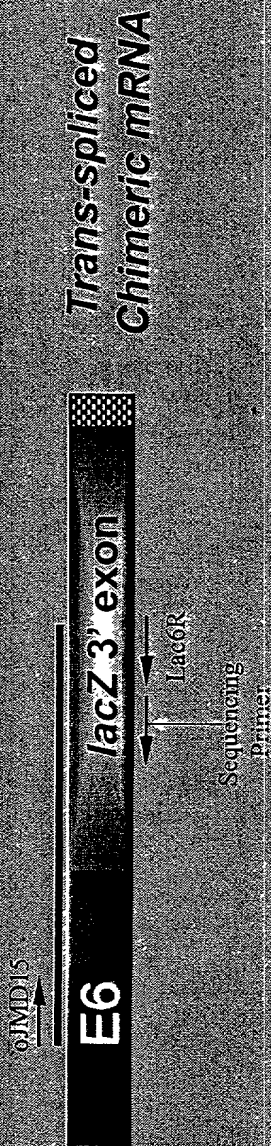


FIGURE 57

INTRONIN

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Trans-splicing in 293 Cells (Co-transfections)



PTM	<u>Binding Domain</u>		<u>% trans-spliced</u>	
	<u>Region</u>	<u>Size (nt)</u>	<u>226 sd</u>	<u>880 sd</u>
HPV-PTM1	A	80	69	0.6
HPV-PTM2	A	149	45	0.9
HPV-PTM5	A+B	140	55	0.8
HPV-PTM5ΔBP/PPT	A+B	140	0.5	0.2
HPV-PTM6	A+B	117	59	1
HPV-PTM8	C	104	7	37
HPV-PTM9	C	174	14	22
CF-PTM27	CF intron	411	0	0

Quantification of *trans*-splicing efficiency using real-time QRT-PCR

FIGURE 58

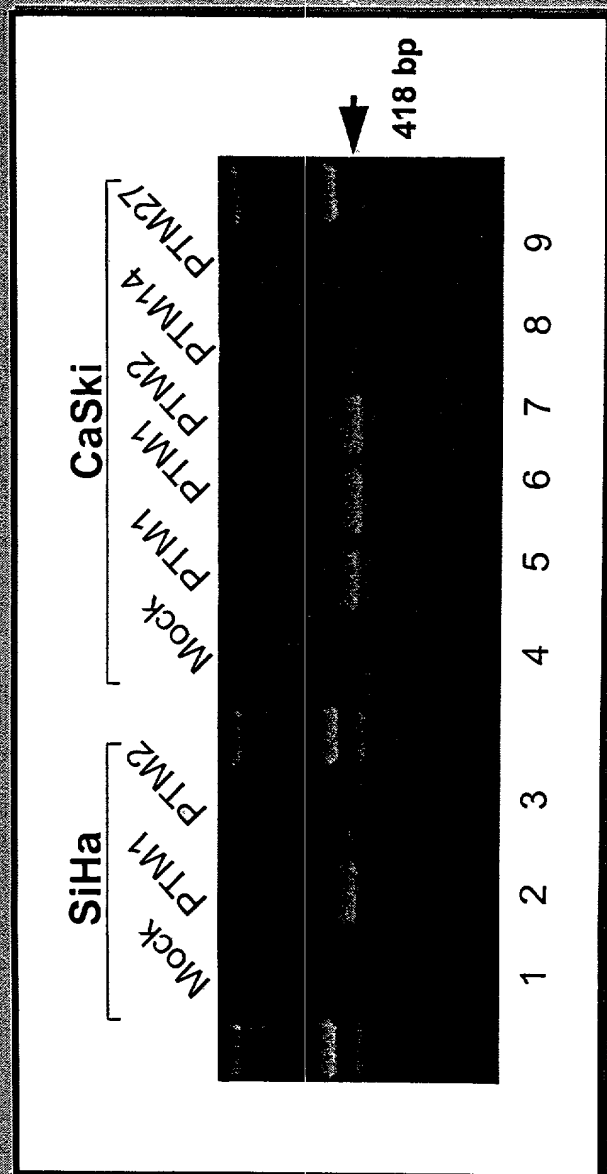
Trans-splicing into Endogenous HPV Pre-mRNA Target in SiHa & CaSki Cells

RT-PCR Conditions

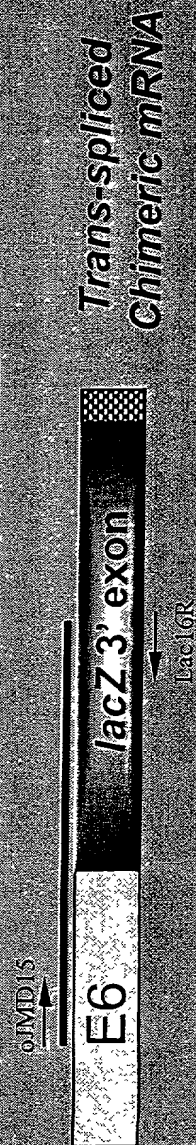
- Total RNA: 400 ng/rxn
- Primer's: oJMD15 + Lac16R
- # Cycles : 35
- Expected product : 418 bp

Details

- PTM1, PTM2 : HPV targeted, specific
- PTM14 : CF targeted, non-specific, has 23 bp BD
- PTM14 : CF targeted, non-specific, has 411 bp BD



SiHa : Single copy
CaSki : ~ 400-500 copy!!

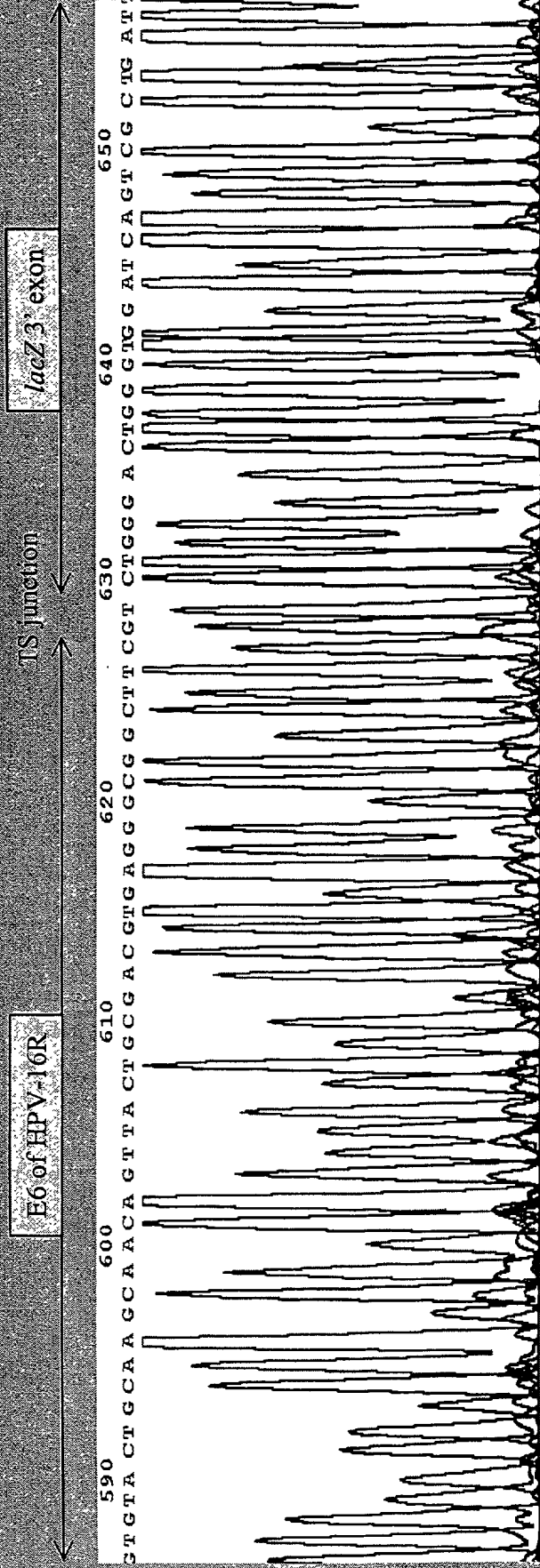
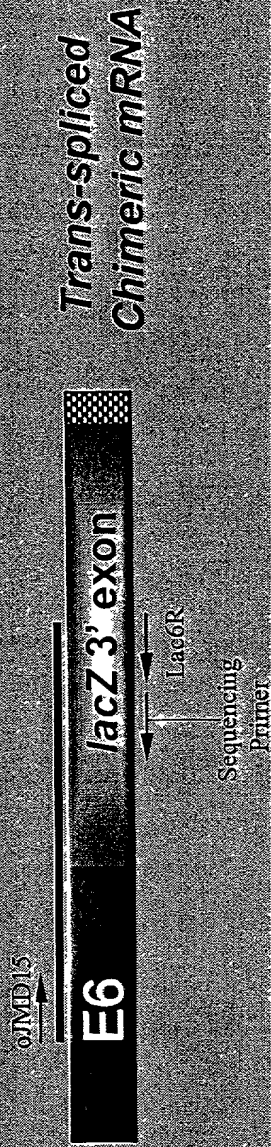


RT-PCR Analysis of total RNA

FIGURE 59

INTRONN

Accurate Trans-splicing of HPV-PTM1 in Si Ha Cells (Endogenous target pre-mRNA)



INTRONN

FIGURE 60

Trans-splicing in SiHa Transfections

(Endogenous target)

<u>PTM</u>	<u>% <i>trans</i>-spliced</u>
pcDNA3.1	0
HPV-PTM1	0.16
HPV-PTM5	0.12
HPV-PTM6	0.11
CF-PTM27	0

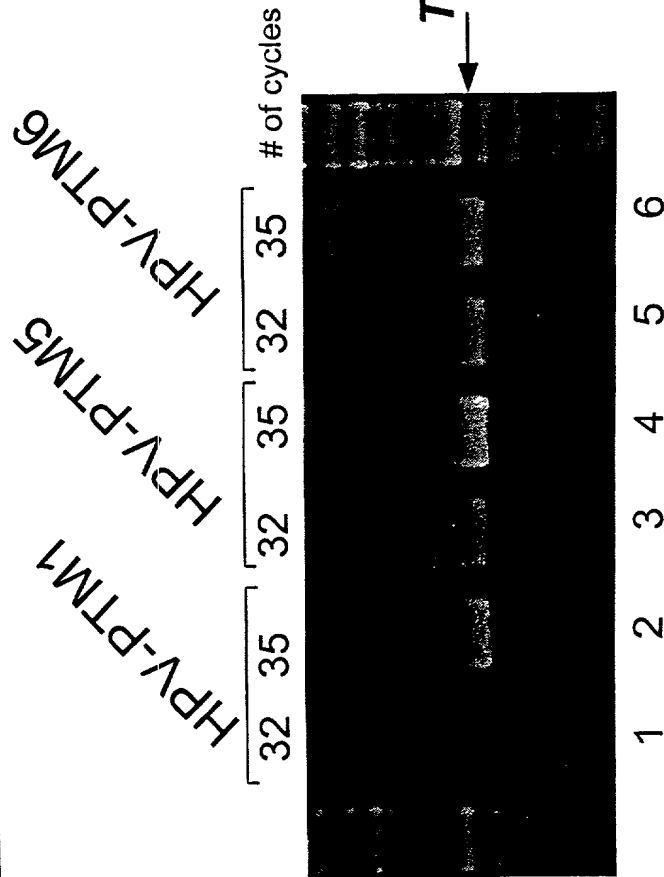
Quantification of *trans*-splicing efficiency using real-time QRT-PCR

INTRONN

FIGURE 61

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Trans-splicing Efficiency of HPV-PTM1, 5, & 6 in SiHa Cells



- SiHa cells transfected with 1.5 µg plasmid DNA, LipoPlus
- RNA isolated after 48 hr
- Total RNA: 500 ng/Rxn
- Primers: oJMD15 + Lac16R
- Expected product: 418 bp

1, 3, 5, : 32 cycles

2, 4, 6, : 35

Deletion of polypyrimidine tract abolishes *trans*-splicing

HPV-PTM5 (has all the elements)



HPV-?PPT (PPT has been deleted)



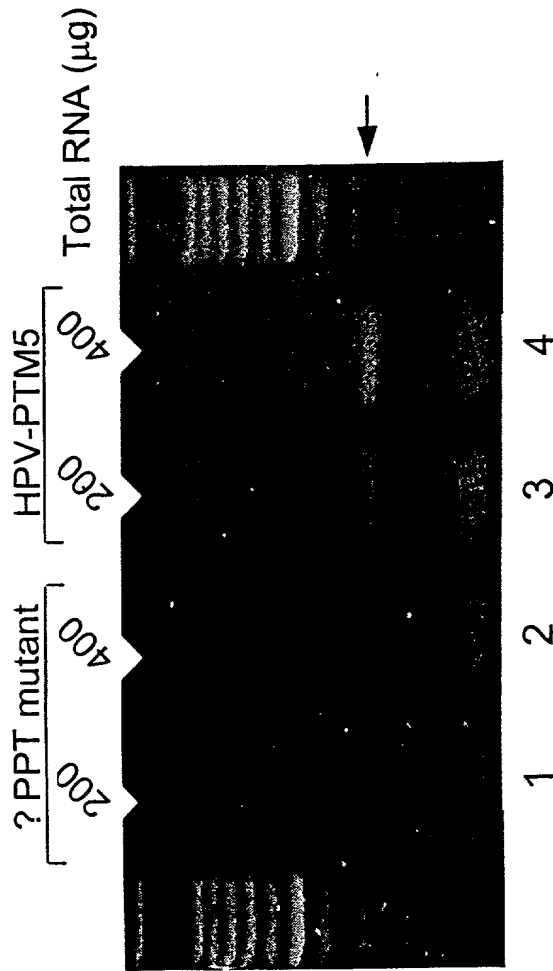
Methods:

- SiHa cells transfected with 1.5 μ g of plasmid DNA
- Total RNA isolated after 48 hr and analyzed by RT-PCR (30 cycles)

Primers: oJMD15+Lac6R
Expected product: 269 bp

Lanes 1 & 2: RNA from cells transfected with HPV-?PPT (mutant); No trans-splicing detected

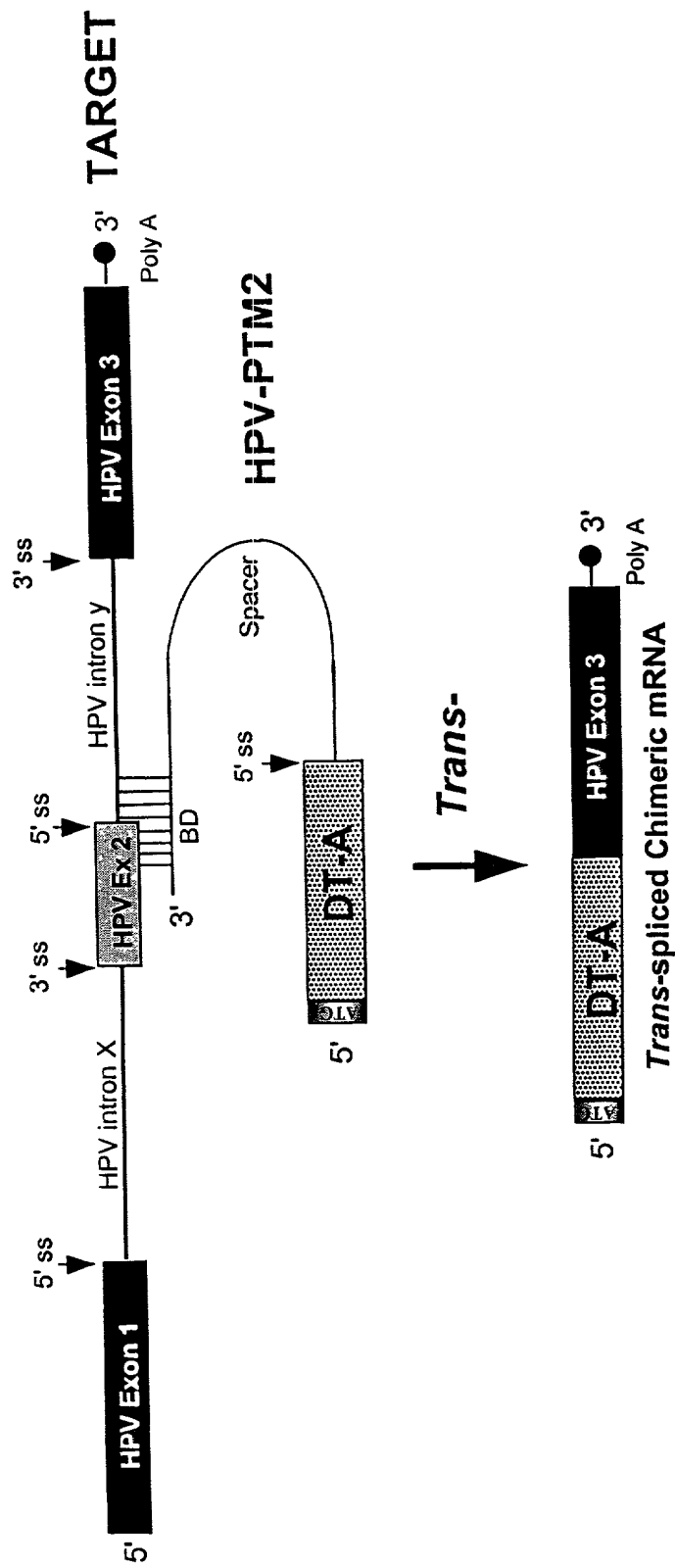
Lanes 3 & 4: RNA from cells transfected with HPV-PTM5 plasmid; trans-splicing Detected (269 bp product)



INTRONN

FIGURE 63

SMaRT Strategy by 5' Exon Replacement



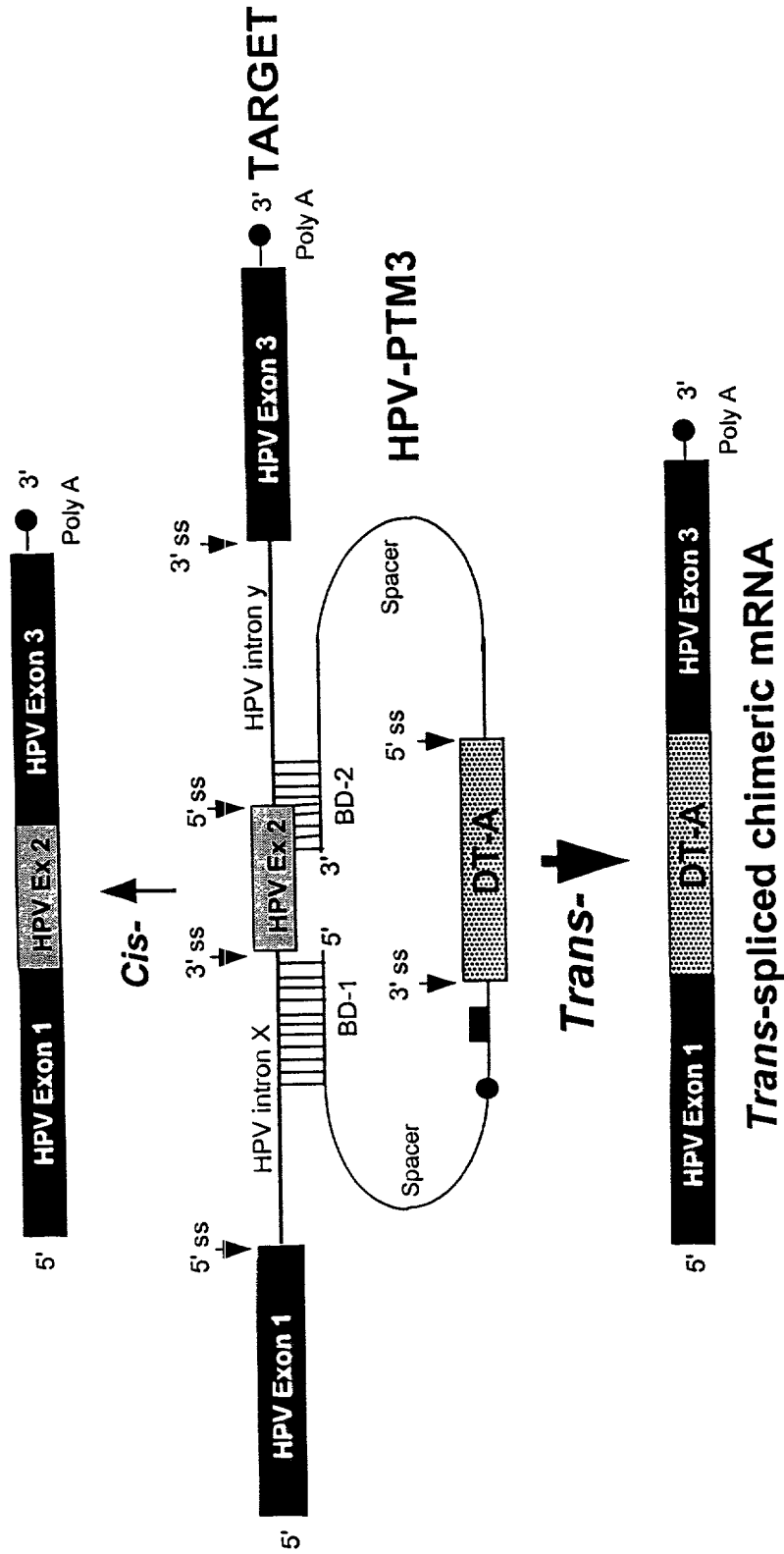
Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

INTRONN

FIGURE 64

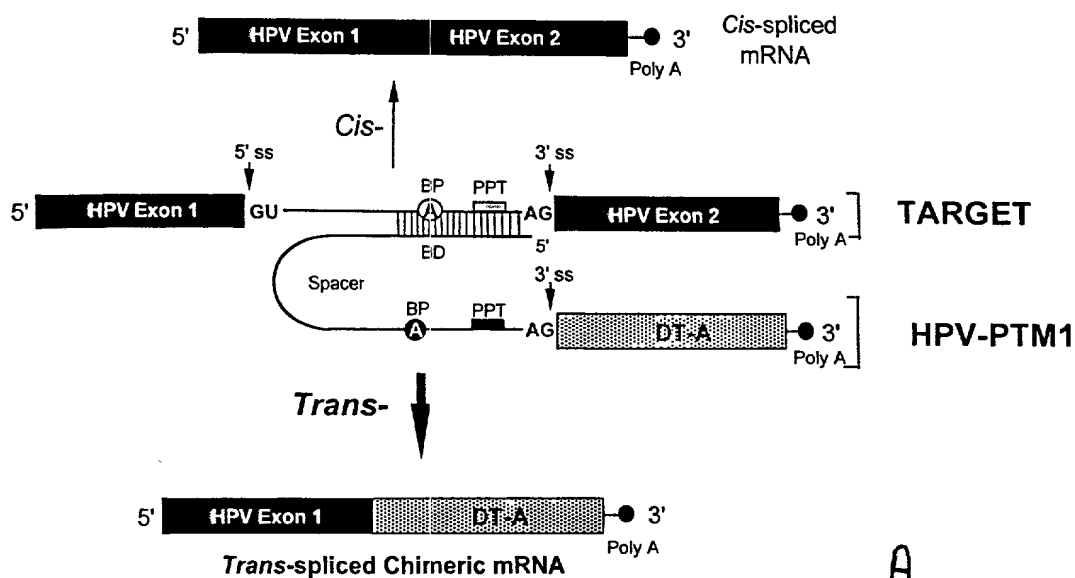
HPV to 92

Double Trans-splicing

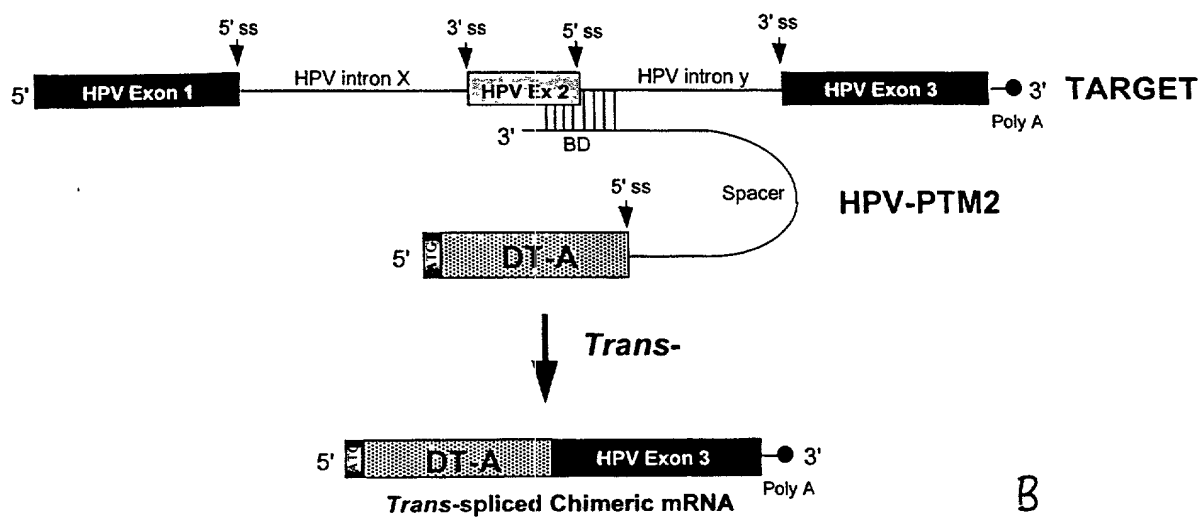


Schematic diagram of a double trans-splicing PTM binding to the 3' and 5' splice sites of the HPV mini-gene target

SMaRT Strategy by 3' Exon Replacement: Schematic diagram of a PTM binding to the 3' splice site of the HPV mini-gene target



SMaRT Strategy by 5' Exon Replacement: Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target



HPV-PTM3 (For Internal exon replacement)

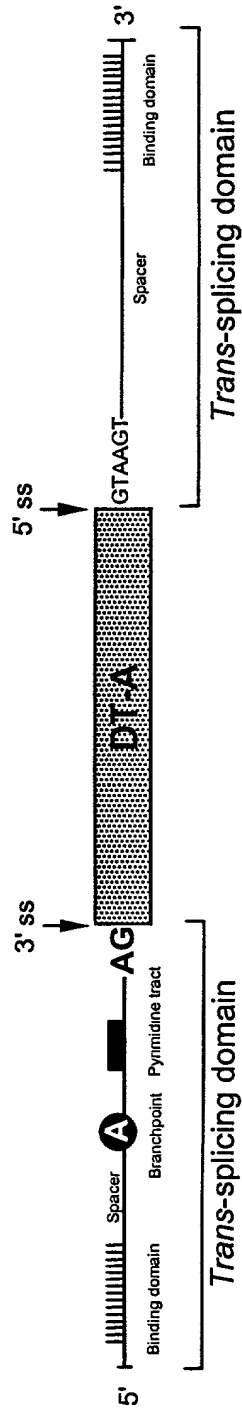


FIGURE 67